Molecular epidemiology of *Mycobacterium tuberculosis* in Madang, Papua New Guinea

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Abbreviations

AMK	Amikacin
BCG	Bacille Calmette-Guérin
CAP	Capreomycin
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeat
DNA	Deoxyribonucleic Acid
DOTS	Directly Observed Treatment, Short-Course
DR	Direct Repeat
DST	Drug Susceptibility Testing
EMB	Ethambutol
ETD	Ethionamide
HIV	Human Immunodeficiency Virus
INH	Isoniazid
IS	Insertion Sequence
KAN	Kanamycin
LSP	Large Sequence Polymorphism
MDR	Multidrug Resistant
MIRU	Mycobacterial Interspersed Repetitive Units
MTBC	*Mycobacterium tuberculosis* Complex
PAS	*p*-Aminosalicylic Acid
PCR	Polymerase Chain Reaction
PNG	Papua New Guinea
PNG IMR	Papua New Guinea Institute of Medical Research
POA	Pyrazinoic Acid
PZA	Pyrazinamide
QMRL	Queensland Mycobacterium Reference Laboratory
RFLP	Restriction Fragment Length Polymorphism
RIF	Rifampicin
RNA	Ribonucleic Acid
SNP	Single Nucleotide Polymorphism
STR	Streptomycin
Swiss TPH	Swiss Tropical and Public Health Institute
TB	Tuberculosis
TST	Tuberculin Skin Test
VNTR	Variable Number of Tandem Repeats
WHO	World Health Organization
XDR	Extensively Drug Resistant

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Summary

In 2010, TB was estimated to have caused 8.8 million incident cases and more than 1.4 million deaths worldwide. The TB-HIV co-epidemic and the development of drug resistances are major challenges for TB control programs. The long duration of anti-TB therapies and the limited treatment options led to the emergence of drug-resistant *Mycobacterium tuberculosis* strains in virtually all regions of the world. Estimates indicated that in 2009, about 3.3% of all new TB patients worldwide had MDR-TB.

PNG belongs to the seven countries in the Western Pacific region with the highest estimated TB burden. Yet, very scarce information is available about the characteristics of the circulating *M. tuberculosis* population in this country. On the basis of a tight collaboration between the Swiss TPH and the PNG IMR, this project was set up to initiate long term TB investigations in PNG.

M. tuberculosis isolates from pulmonary TB patients in Madang and surroundings were characterized with focus on drug resistance and strain genotypes. Apart from the molecular analyses conducted mainly in Switzerland, this project also aimed at transferring to PNG some technical knowledge necessary for the improvement of TB control in that region.

M. tuberculosis isolates were collected during a pilot study conducted in 2005-2007 and in the frame of an ongoing treatment cohort study initiated in 2009. DST indicated substantial rates of drug resistance, further confirmed by the presence of specific resistance-conferring mutations; in particular, 5.2% MDR-TB was observed. SNP-based genotyping showed that out of the six *M. tuberculosis* lineages described worldwide, the strains from Madang and surroundings predominantly belonged to the Euro-American and East-Asian lineages (Lineage 4 and Lineage 2, respectively). Furthermore, Lineage 2 strains were significantly associated with drug resistance. Molecularly clustered strains were identified using a combination of two discriminatory genotyping methods and suggested evidence for patient-to-patient *M. tuberculosis* transmission, including transmission of drug-resistant strains.

This study provides the first molecular description of the *M. tuberculosis* strains circulating in the Madang area of PNG. Nevertheless, to elucidate the burden of TB at the country-level, further studies taking into account several locations throughout PNG are needed.

Résumé

L'Organisation mondiale de la Santé estime qu'en 2010, il y a eu 8,8 millions de nouveaux cas de tuberculose dans le monde ainsi qu'environ 1,4 millions de décès dus à cette maladie. Les deux principaux défis auxquels la lutte contre la tuberculose doit faire face sont d'une part l'apparition de souches résistantes aux médicaments et d'autre part la co-épidémie avec le virus du SIDA. En effet, la durée du traitement ainsi que le choix limité de médicaments à disposition ont permis à des résistances de se développer dans toutes les régions du monde. Les estimations de 2009 indiquent que 3,3% des patients nouvellement infectés étaient porteurs d'une souche multi-résistante de *Mycobacterium tuberculosis* (l'agent causatif de la tuberculose).

La Papouasie Nouvelle Guinée fait partie des sept pays du Pacifique enregistrant le plus de cas de tuberculose. Pourtant, très peu d'information existe concernant les caractéristiques des souches de *M. tuberculosis* circulant dans ce pays. Dès lors, ce projet a été initié dans le cadre d'une collaboration entre l'Institut Tropical et de Santé Publique Suisse et l'Institut de Recherche Médicale de Papouasie Nouvelle Guinée, afin d'étudier l'actuelle épidémie de tuberculose dans ce pays.

Des souches de *M. tuberculosis* isolées de patients souffrant de tuberculose pulmonaire dans la région de Madang ont été caractérisées, dans le but d'identifier leur génotype ainsi que l'éventuelle présence de résistances aux médicaments. Au delà des analyses moléculaires principalement effectuées en Suisse, ce projet avait aussi pour but de transférer en Papouasie Nouvelles Guinée les technologies de base nécessaires au contrôle de la tuberculose.

Les échantillons ont été collectés lors d'une étude pilote effectuée en 2005-2007 ainsi que dans le cadre d'une étude de cohorte initiée en 2009 dans la région de Madang. La détermination phénotypique des résistances aux médicaments a montré un taux élevé de résistance confirmé par la présence de mutations spécifiques. En particuliers, 5.2% des souches étaient multi-résistantes, c'est-à-dire résistantes aux deux meilleurs médicaments actuellement disponibles pour le traitement de la tuberculose. Le génotypage par identification de mutations spécifiques a montré que les souches de la région de Madang appartenaient principalement à deux des six lignées de *M. tuberculosis* décrites dans le monde: la lignée euro-américaine et la lignée de l'est asiatique. Par ailleurs, nous avons observé que la lignée de l'est asiatique était plus fréquemment résistante aux médicaments. En combinant deux méthodes de typage moléculaire particulièrement

discriminatoires, les souches partageant le même génotype ont pu être identifiées. Ces groupes de souches suggèrent qu'il y a certainement eu transmission de *M. tuberculosis* d'un patient à l'autre, y compris transmission de souches résistantes.

Cette étude fournit la première description moléculaire des souches de *M. tuberculosis* circulant dans la région de Madang en Papouasie Nouvelle Guinée. Toutefois, afin de mieux comprendre l'ampleur de l'épidémie au niveau national, des études complémentaires comparant diverses régions du pays seraient nécessaires.

Introduction

1. History of tuberculosis

Tuberculosis (TB) already existed long ago and is virtually present on all continents in the world. Previously known as white plague (reflecting the paleness of TB patients), consumption (TB patients literally consumed from their inside), wasting disease or phtisis (the Greek equivalent for "wasting"), TB has possibly caused more human deaths than any other microbe [1]. The term phtisis has been found in documents dating from classical Greece, in the 4th century before Christ. Paleopathologists could show signs of TB in early civilization times on the basis of typical bone or spinal lesions (Pott's disease), as well as by the molecular analysis of ancient deoxyribonucleic acid (DNA). Samples collected from Pre-Columbian [2,3] and Egyptian [1,4] mummies indicated the presence of TB already about 5,000 years ago. Phylogenetic analyses showed that tubercle bacilli were contemporaneous of early hominids in East Africa about 3 million years ago, and have thus been co-evolving with their human hosts much longer than previously thought [5]. Out of Africa migrations of modern humans (*Homo sapiens*) accompanied by *Mycobacterium tuberculosis* are believed to have initiated the spread of TB to other parts of the world about 60,000-40,000 years ago [5–7].

In 1819, the French physician René Laennec, inventor of the stethoscope, described the symptoms of TB, and with him started the modern understanding of the disease. TB was shown to be infectious in 1865 by Jean-Antoine Villemin, who successfully inoculated a rabbit with some liquid taken from a tuberculous cavity. On 24 March 1882, Robert Koch showed in a lecture that TB was caused by the tubercle bacillus, which he could visualize using a specific staining method of his invention. This famous date is still commemorated nowadays as World TB Day.

Inspired by Louis Pasteur's cure for rabies, Robert Koch also described tuberculin in 1890, an extract of tubercle bacillus cells, which later became used as a diagnostic tool, still known today as Mantoux test [8]. In 1905, Robert Koch was awarded the Nobel Prize for having elucidated the etiology of TB. In 1895, X-rays were discovered by Wilhelm Röntgen and rapidly contributed to the diagnosis of TB. Following Robert Koch's work on tuberculin, Clemens von Pirquet described latent TB in 1907, when patients were reacting to tuberculin in the absence of TB symptoms. The use of

cannulated needles to inject tuberculin was introduced in 1908 by Charles Mantoux, who gave his name to the test [1].

Since the mid-19th century, TB started to decline. Although not fully explained, this decline can certainly be attributed to various factors including better living conditions, nutrition, hygiene, as well as less frequent contacts between individuals [1,9,10]. In the same period, sanatoria opened in remote regions for the convalescence of TB patients in an isolated environment. In 1921, the first anti-TB vaccine was developed by Albert Calmette and Camille Guérin. The attenuated strain called "Bacille Calmette-Guérin" (BCG) was obtained after *in vitro* passaging of a bovine TB strain (*M. bovis*) until loss of virulence. BCG remains the only available vaccine against TB nowadays, although its efficacy against pulmonary TB in adults varies from 0 to 80% [11]. A great achievement in TB control was made in the 1940s, when the first anti-TB drugs were found (see section 5).

2. Global burden of tuberculosis

After human immunodeficiency virus (HIV), TB is the second cause of human death from an infectious disease worldwide [12]. In 2010, the World Health Organization (WHO) estimated that 8.8 million people were newly infected with TB and 1.4 million died of TB [12]. In other words, TB caused about 3,800 deaths per day in 2010. Although the TB incidence has been decreasing in the last years, the absolute number of cases remains high as a result of the global population growth. Figure 1 depicts the worldwide TB incidence in 2010.

TB is a poverty-related disease with most cases occurring in Asia and Africa [12]. According to estimates from 2010, the 22 countries with the highest TB burden account for about 80% of the worldwide number of cases, with the largest numbers of incident cases seen in India, China, South Africa, Indonesia and Pakistan [12].

HIV and TB coexist in many regions and form together a co-epidemic. TB is the leading cause of death among HIV-infected people, accounting for a fourth of HIV deaths worldwide [13]. Due to their often impaired immune system, TB patients co-infected with HIV are 21 to 34 times more likely to develop active TB disease than HIV negative individuals [13]. About 13% of all TB incident cases were HIV infected in 2010, and of them approximately 82% were from Africa [12,13]. In 2008, South Africa alone accounted for 24% of all new HIV-TB cases worldwide, even though its estimated population comprised less than 1% of the global population [14].

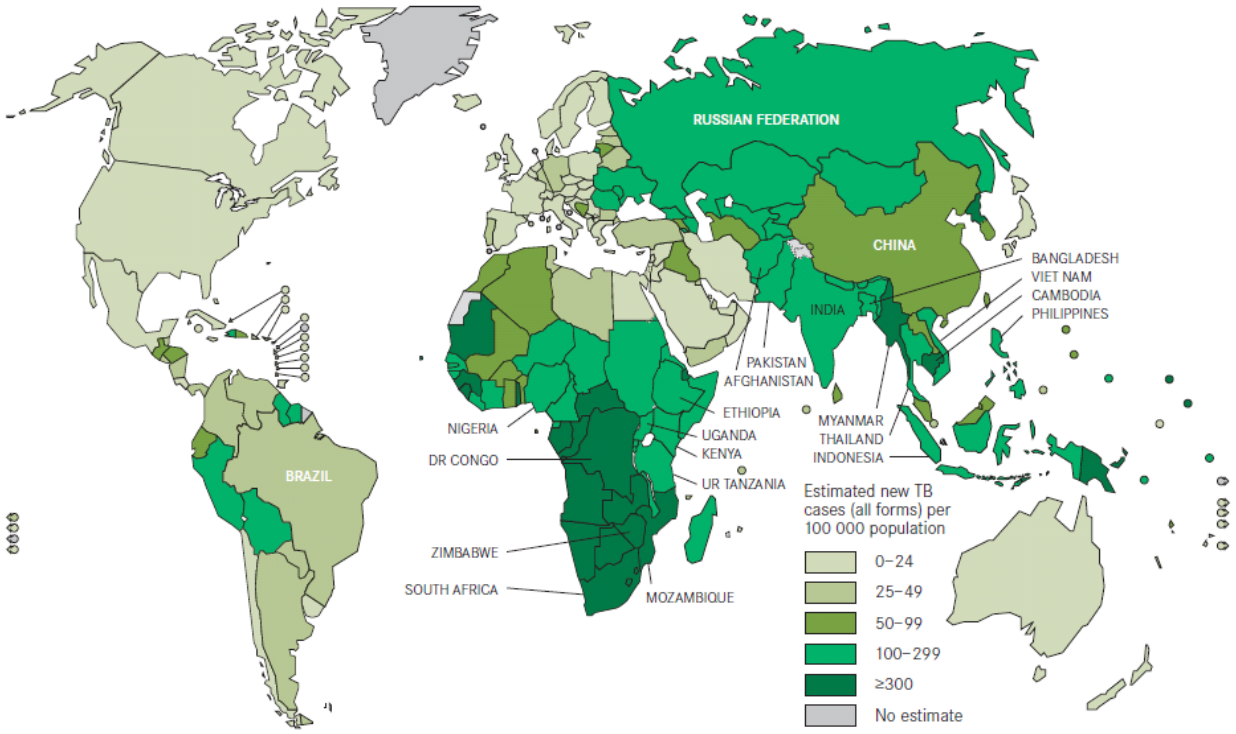

Figure 1: The estimated rate of TB incidence by country in 2010 [12].

Together with HIV, the emergence of drug resistance is another major aspect contributing to the current burden of TB worldwide. Globally, it is estimated that 3.3% of all new TB cases have multidrug resistant TB (MDR-TB) [15], which requires specific second-line drugs with a lower treatment success rates (see section 5). However, only about 16% of MDR-TB patients are estimated to have received treatment in 2010 [16], and because the identification of drug resistance is not routinely available everywhere, these numbers are likely to be underestimated. The highest rates of MDR-TB are reported from Eastern Europe and Central Asia [17]. However, due to the number of inhabitants, the highest numbers of MDR-TB cases are seen in China and India, accounting together for over 50% of the global MDR-TB burden [17]. In 2008, the WHO reported 150,000 deaths caused by MDR-TB globally [15]. Extensively drug resistant TB (XDR-TB), for which virtually no treatment is available, was reported in an increasing number of countries (69 by end 2010), mostly in Eastern Europe (former Soviet countries), Central Asia and South Africa [15].

Since 1994, the directly observed treatment – short-course (DOTS) strategy has been launched by the WHO as an effective and affordable approach to detect, treat and control TB [18]. The DOTS strategy comprises five components: 1) political commitment, 2) case detection by bacteriology, 3)

standardized treatment with supervision, 4) effective drug supply, and 5) treatment monitoring. In 2006, the Stop TB Strategy added new elements to DOTS in order to better address the TB-HIV co-morbidities and the emergence of drug resistance [19].

While the Stop TB Strategy targets to halve the number of deaths from TB by 2015 compared to 1990 values, the Millenium Development Goal aims to halt and reverse the global TB incidence by 2015 (also in comparison with 1990). By 2050, the Stop TB Strategy aim is to eliminate TB as a public health problem [19].

3. Pathogenesis and disease

TB is a disease caused by members of the *M. tuberculosis* complex (MTBC), a group of Gram-positive bacteria characterized by 99.9% similarity at the nucleotide level and identical 16S rRNA sequences [20]. MTBC members (also referred to as "ecotypes") include the human pathogens *M. tuberculosis* and *M. africanum*, as well as *M. bovis* (infecting cattle), *M. caprae* (infecting goats and sheep), *M. microti* (infecting voles) and *M. pinnipedii* (infecting marine mammals) [20]. In humans, the result of *M. tuberculosis* infection can vary between latent TB (no clinical symptoms and no transmission) and active TB disease, which can be pulmonary or extrapulmonary. Ninety percent of *M. tuberculosis* infections remain in a latent stage, whereas 10% will develop into active TB at some point during a person's lifetime [21].

Life cycle

Transmission of TB occurs by the inhalation of infectious aerosols emitted by active pulmonary TB patients. After inhalation of a droplet containing a few *M. tuberculosis* bacilli, the bacteria will be phagocytosed by alveolar macrophages in the lungs, where the bacilli will settle in the case of pulmonary TB. Occasionally, the bacilli will be further transported to other parts of the human body and will cause extrapulmonary TB. *M. tuberculosis* survival in the host starts with its capacity to persist in alveolar macrophages by preventing the phagosome maturation [22].

In the lungs, the formation of granulomas (or "tubercles", the hallmark of TB) is initiated by the cytokine-mediated recruitment of additional macrophages, dendritic cells and lymphocytes to the site of infection [23]. In the granuloma, the bacilli and the immune system initiate a dynamic cross-talk balancing between infection control and disease [24]. The granuloma is first constituted of disorganized macrophages, monocytes, and neutrophils but it becomes more organized when

lymphocytes are recruited, with macrophages grouped in the center and lymphocytes at the periphery. This order of infiltration reflects the successive roles of innate and acquired immunity following *M. tuberculosis* infection [24]. The granuloma becomes vascularized, but as it matures, the vessels reduce while the fibrous outer layer thickens [25]. In late stages of the granuloma formation, *M. tuberculosis* can enter a low-replicative phase in the hypoxic caseous center of the granuloma. This low-replicative stage corresponds to latent TB. However, there is increasing evidence that the dichotomy between latent and active TB is a too simplified view. In fact, *M. tuberculosis* sub-populations in various replicative stages can be found in granulomas, with a spectrum of presentations (sterile tissue, caseous hypoxic lesions or liquefied cavities of replicating bacilli) [21,23,26]. TB can remain in a latent state for decades in equilibrium between *M. tuberculosis* replication and host defense, mediated by the complex interplay of immune cells and inflammatory molecules. Figure 2 depicts the granuloma formation.

Despite the apparent strong immune response which contains the *M. tuberculosis* infection in the granuloma, the bacilli are yet not fully eliminated. Ultimately, latent TB converts into active disease when cavitary lesions develop and the number of bacilli increases in the caseous center of the granuloma. Live bacilli can reach the alveoli once the granuloma center collapses and the patient becomes infectious [25]. Viable and infectious bacilli are freed into the airways, resulting in a productive cough spreading infectious bacilli in the air. The life cycle continues when other persons get infected. It is estimated that a single person with active TB can infect up to 45 other individuals [27]. Active pulmonary TB is characterized by unspecific symptoms, principally prolonged and productive cough (> two weeks), shortness of breath, chest pain, hemoptysis (expectoration of blood), night sweats, weight loss, or fever [28].

Acute TB, when disease develops directly after infection without previous latent stage or granuloma formation is rare [22]. In adults, active TB typically results from the reactivation of existing latent TB, rather than as direct outcome of primary infection. Generally, the immediate onset of disease following infection only occurs in immunocompromised individuals, such as newborns, elderly or HIV-infected patients [22]. In fact, the conditions influencing the progression to active TB are not fully understood [25], although factors such as HIV and diabetes are known to favor a prompter development of active TB [21,29].

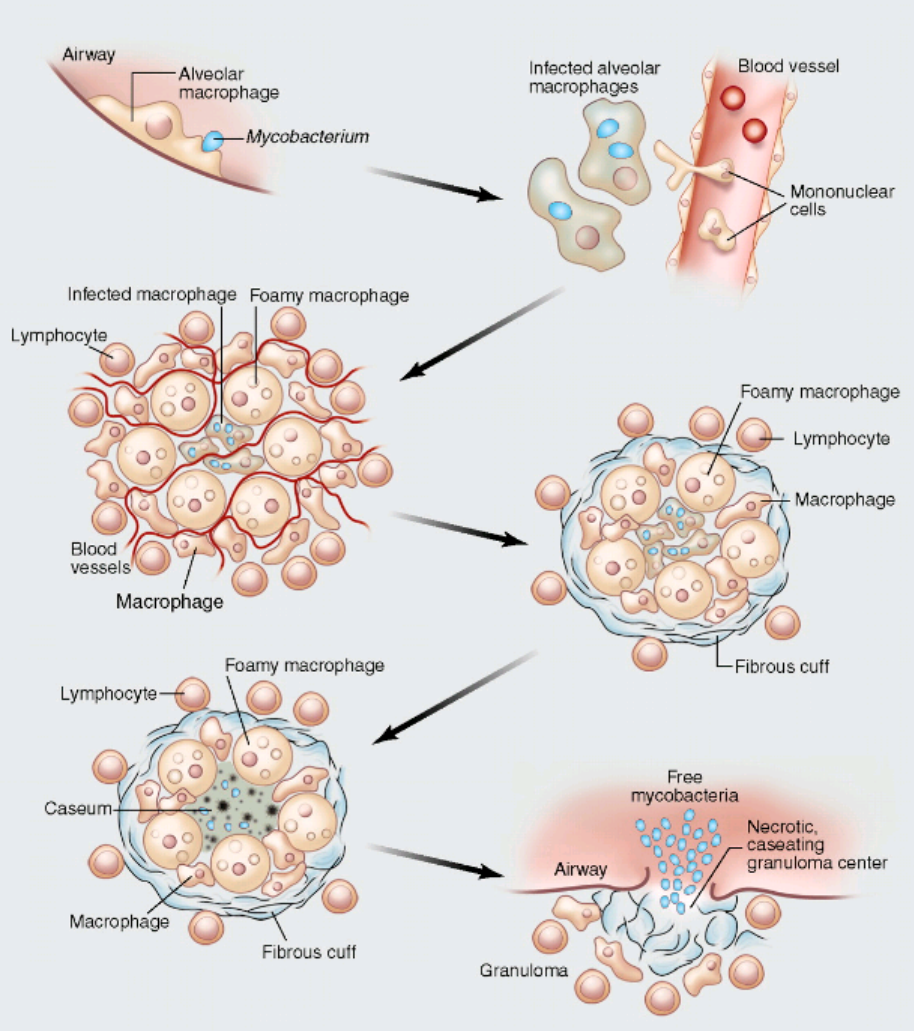

Figure 2: Life cycle of *M. tuberculosis* and granuloma formation [23].

4. Diagnosis

Smear microscopy

The most widely used diagnosis for active pulmonary TB is the examination of sputum smear by microscopy after Ziehl-Neelsen staining. The Ziehl-Neelsen staining method relies on the specific lipid rich cell wall of mycobacteria, which retains the red carbol fuchsin dye after alcohol-acid decolourization [30]. This is the reason why *M. tuberculosis* is often referred as "acid-fast bacillus". Figure 3 depicts the structure of *M. tuberculosis* cell wall.

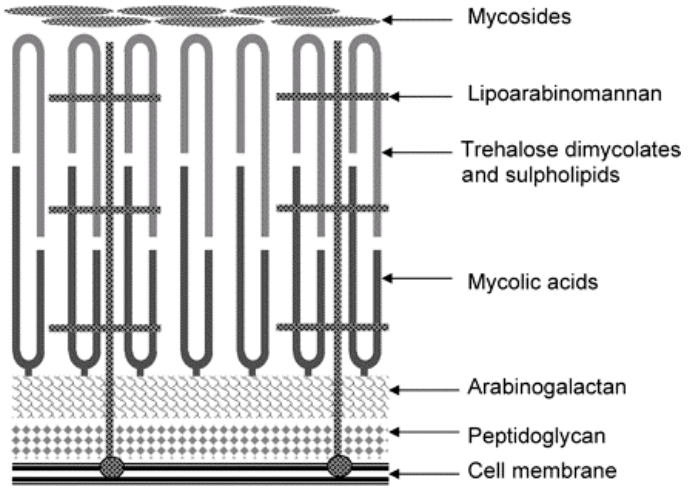

Figure 3: Schematic representation of the *M. tuberculosis* cell wall [31].

Sputum smear examination by light microscopy is a highly specific, fast and cheap method, and often the only TB diagnosis available, especially in resource-limited settings. Unfortunately, the sensitivity of smear microscopy is variable, with 20 to 80% of *M. tuberculosis* infections missed by this method [32]. Apart from the low sensitivity, the main drawback of sputum smear microscopy is the intrinsic reliance on sputum production, limiting its use in children and HIV positive patients [33,34]. TB-HIV patients produce little sputum and are often smear-negative due to a reduced pulmonary cavity formation and a low sputum bacillary load, challenging the diagnosis of TB in this population [34].

Fluorescent microscopy is a faster, more sensitive, but less specific alternative, using acid-fast fluorochrome dyes instead of Ziehl-Neelsen staining [35–37]. In 2011, the WHO recommended to initiate the implementation of fluorescent microscopy with a light-emitting diode (LED) for the diagnosis of TB [32].

Chest radiography

Chest radiography (X-rays) is often used as a complement to smear microscopy to diagnose TB, especially in sputum negative patients. Typical TB-related abnormalities in the lungs (infiltrations, nodules or cavities) can be recognized by trained medical personal but are generally not used as the only definite TB diagnosis [28].

In vitro culture

Bacterial cultures *in vitro* can provide a definitive TB diagnosis and is currently considered as the gold standard method. Unfortunately, *M. tuberculosis* grows very slowly, and with a doubling

time of about 20 hours (depending on the growth medium used), TB diagnosis by culture can take 3-8 weeks [30]. The sensitivity is high as only a few viable bacilli are sufficient to initiate growth, but sputum samples must be decontaminated before culture inoculation to prevent overgrowth by other faster growing microorganisms. However, decontamination can also be harmful to mycobacteria and therefore, culture-based diagnosis is not a 100% sensitive method [38]. The main drawback of *M. tuberculosis* culture is the requirement for high biosafety laboratory facilities ("biosafety level 3") and specifically trained staff to avoid aerosol transmission. Such facilities are very costly and often not available in resource-limited settings.

Alternative diagnostic tools

Molecular diagnostic tests based on DNA amplification have been proposed to complement (but not replace) the conventional bacteriological diagnosis of TB [39]. Most of these tests have the advantage to allow the rapid detection of *M. tuberculosis* and are highly specific, although their sensitivity often remains sub-optimal [39]. Nevertheless, newer assays are considerably more sensitive and recently, a fully automated system called Xpert MTB/RIF (Cepheid, USA) has been validated. Xpert MTB/RIF detects *M. tuberculosis* infections and rifampicin (RIF) resistance in less than two hours, directly from sputum, regardless of the smear status, and with high sensitivity [40–42]. This method is based on a real-time polymerase chain reaction (PCR) amplifying specific regions of the *rpoB* gene. Furthermore, it requires virtually no specific infrastructures since all reagents are contained in a disposable cartridge [41].

The tuberculin skin test (TST), traditionally used to identify latent *M. tuberculosis* infections, is based on the measurement of the inflammatory reaction, which follows the intradermal injection of tuberculin [30]. Despite its usefulness and simplicity, the TST is limited by its poor specificity, particularly in BCG vaccinated populations [43]. In the past decade, commercial antigen-specific assays measuring interferon-gamma released from T lymphocytes by enzyme-linked immunoassay (QuantiFERON Gold, Cellestis, Australia) and enzyme-linked immunospot (T-SPOT.*TB*, Oxford Immunotec, UK) have been developed. Both are based on antigens specific to *M. tuberculosis*, such as the early secretory antigenic target 6 (ESAT6) and culture filtrate protein 10 (CFP10), which are absent from BCG strains [44]. Unfortunately, these tests cannot distinguish latent from active *M. tuberculosis* infections and cross-reaction with other mycobacteria cannot be excluded [30,43]. Another limitation of these methods is the need for sophisticated instruments and viable white blood cells, which limits their implementation almost exclusively to developed countries [34].

5. Treatment and drug resistance

In the absence of drug resistance, *M. tuberculosis* can successfully be treated by chemotherapy since the 1940s, when streptomycin (STR) and *p*-aminosalicylic acid (PAS) were first discovered, later followed by isoniazid (INH) and RIF in 1952 and 1963, respectively. Table 1 lists the first-line and second-line drugs currently available for the treatment of TB.

According to the WHO recommendations [28], national TB programs should have three standard regimens for TB treatment: i) for new TB patients, ii) for previously treated patients supposedly not MDR, and iii) for MDR patients.

The standard regimen for new pulmonary TB patients consist of two months intensive phase with daily INH, RIF, pyrazinamide (PZA) and ethambutol (EMB), followed by a four-month continuation phase with daily INH and RIF [28].

Previously treated patients ($\geq$ 1month of anti-TB drugs in the past) are globally five times more often MDR and should therefore be treated according to drug susceptibility testing (DST) results, if available [45]. If DST is not available and the patient returns after default or relapse (low likelihood of MDR), an 8-month regimen should be prescribed, based on first-line drugs and additional STR for the first two months (intensive phase). If the returning patient has a history of treatment failure (high likelihood of MDR) but no DST is available, empirical MDR treatment should be initiated [28].

For the treatment of MDR-TB, three main groups of drugs are available: 1) Injectable aminoglycosides: STR, kanamycin (KAN), amikacin (AMK) or capreomycin (CAP); 2) Fluoroquinolones: ofloxacin, levofloxacin or moxifloxacin; 3) Oral bacteriostatic drugs: ethionamide (ETD), PAS or cycloserine. The regimen for MDR cases should consist of at least four drugs, if possible with DST-proven susceptibility. If any first-line drug is likely to be still effective, it should be included in the regimen, as they are more potent and have less adverse effects than second-line drugs. In addition, all MDR patients should receive one injectable drug (group 1, preferentially AMK or KAN, since STR resistance among MDR-TB is frequent), one fluoroquinolone (group 2), and group 3 drugs in order to obtain a regimen comprising four drugs. After at least 6 months of intensive phase, the injectable drug can be discontinued. The overall duration of MDR-TB treatment should be of at least 18 months after sputum conversion. The same principles apply to XDR-TB patients [28].

Table 1: Most common drugs used for the treatment of TB.

Drug name	**Year of discovery**	**Description** [28,46]	**Mode of action**
Isoniazid	1952	INH (hydrazide of isonicotinic acid) is highly bactericidal against replicating tubercle bacilli.	INH is a prodrug activated by the catalase-peroxidase KatG; active INH binds the enoyl-acyl carrier protein reductase InhA, which elongates mycolic acid for the cell wall [47].
Rifampicin	1963	RIF is a semi-synthetic derivative of rifamycin with bactericidal action against tubercle bacilli.	RIF binds to the RNA polymerase β-subunit, which inhibits RNA synthesis and protein translation [47,48].
Pyrazinamide	1954	PZA is weakly bactericidal but active against slow-replicating bacilli in acidic lesions. It shortens treatment duration and reduces risks of relapse.	PZA is a prodrug converted into pyrazinoic acid (POA) by a pyrazinamidase [46]. POA disturbs the membrane proton gradient and membrane transport. PZA also inhibits the ribosome-sparing process of *trans*-translation by binding to RpsA [49].
Ethambutol	1961	EMB is a synthetic 1,2-ethanediamine used to shorten therapy and prevent the emergence of resistance.	EMB inhibits the polymerization of the cell wall component arabinan by disrupting the arabinosyl transferase [46,50].
Streptomycin	1943	STR is an aminoglycoside also used against other Gram-negative bacteria. It is administrated by injection and bactericidal, particularly in lung cavities.	STR targets the 30S ribosome subunit and binds to the 16S rRNA and the protein S12 [47,51].
Kanamycin–Amikacin	1957	KAN and its derivative AMK are aminoglycosides. They both need to be injected.	KAN and AMK inhibit protein synthesis by binding the 30S ribosome subunit and modifying the ribosomal structure [46].
Capreomycin	1960	CAP is a macrocyclic peptide antibiotic. It is administrated by injections.	CAP interferes with protein translation by blocking the formation of the 30S ribosome subunit [52].
Fluoroquinolones	1980	Fluoroquinolones are synthetic broad-spectrum antibiotics, which can penetrate into macrophages.	Fluoroquinolones interfer with DNA replication by inhibiting the DNA gyrase (topoisomerase II) and preventing DNA supercoiling [46].
Ethionamide	1956	ETD (2-ethylisonicotinamide) is a derivative of isonicotinic acid. It is a structural analog of INH and also targets InhA.	ETD is a prodrug activated by EtaA/EthA (mono-oxygenase). Like INH, it inhibits InhA, necessary for the mycolic acid synthesis [46].
p-aminosalicylic acid	1948	PAS has a low effectiveness and is poorly tolerated, therefore the last-choice among the second-line drugs [53].	PAS interferes with folic acid and iron metabolisms [46,54].
Cycloserine	1955	Cycloserine is bacteriostatic. It is an effective agent but the severe toxicity limits its use.	Cycloserine blocks enzymes of the cell wall biosynthesis [53].

To ensure a good adherence to treatment, the DOTS program recommends that drugs are taken under the supervision of a designated relative or health worker ("directly observed treatment" – DOT). In some places, patients may come back to the clinic or health office on a daily basis to take their drugs (and have their injections done, if necessary). Furthermore, the DOTS program recommends that all patients are monitored monthly in order to assess their response to the given treatment. This includes reporting symptoms and drug adverse events, re-adjusting the drug dosages if necessary, and controlling the good adherence to treatment. Sputum monitoring by smear microscopy should also be scheduled, in principle at least after two months (at the end of the intensive phase), five months and 6 months of treatment [28].

Definition of drug resistance

Drug resistance can be classified in two categories reflecting the way how resistance emerged: "primary resistance" or "acquired resistance". Primary resistance is defined for patients who have never been previously treated for TB (new TB cases) and have been infected after the transmission of an already drug-resistant strain. In contrast, acquired resistance is defined when drug resistance develops within the patient's body, during the course of treatment. Various factors can promote the acquisition of drug resistance among previously treated cases: poorly supervised treatment, inadequate regimen, availability of drugs without the need for physician prescription, or poor drug quality [55].

Regardless of how drug resistance emerged, two combinations of resistance to more than one drug are particularly critical for the cure of the patient: MDR-TB and XDR-TB. MDR-TB is defined for resistance to at least RIF and INH, the two most potent anti-TB drugs. XDR-TB is defined when MDR-TB cases are additionally resistant to at least one injectable drug (AMK, KAN or CAP) and one fluoroquinolone. MDR-TB cannot be treated with the standard six-month regimen based of first-line drugs, but requires two years or more on treatment with second-line drugs that are generally less potent, more toxic and 50 to 200 times more expensive than first-line drugs [17]. XDR-TB can emerge when second-line drugs are not optimally used during the treatment of MDR-TB. The WHO reports that in average, about 5% of MDR-TB cases were found to have XDR-TB [17]. This shows that for a good control of drug-resistant TB, the prompt identification of drug resistance and initiation of adequate treatment with second-line drugs is crucial to give the patients a better chance of cure and prevent the further development or spread of resistance [17].

Identification of drug resistance

Ideally, DST should be performed for all patients before treatment initiation, so that the most appropriate therapy for each individual can be determined. The gold standard method for DST, also referred to as "phenotypic DST", is based on the proportion method and consists of monitoring mycobacterial growth in a culture medium containing the drug to be tested at a so called "critical concentration" [56]. The proportion method is currently the method of choice to assess drug resistance, but remains criticized due to the fact that *in vitro* conditions might not reflect the patient's body, and therefore not necessarily predict the actual risk of treatment failure [57,58]. As for TB diagnosis, the main drawbacks of culture-based methods is the time required to grow *M. tuberculosis* (3-8 weeks) and the need for biosafety level 3 laboratories as well as specifically trained personal.

An alternative to classical phenotypic DST is the use of molecular markers for the identification of drug resistance. Drug resistance conferring mutations have been described and can be screened to identify drug-resistant strains. A list of genes harboring drug resistance associated mutations is given in table 2.

Table 2: Genes harboring mutations associated with resistance to anti-TB drugs.

Drug name	**Commonly mutated genes and mechanisms of resistance** [46,59]
Isoniazid	*katG* (catalase peroxidase): mutations in *katG* reduce catalase activity and cause high-level INH resistance. Frequent mutation: codon 315. *inhA* promoter (enoyl-acyl carrier protein reductase): mutations in *inhA* promoter enhance target expression and cause low-level INH resistance. Frequent mutations: nucleotides -8 and -15 in the promoter. *ahpC* promoter (alkyl hydroperoxidase): mutations in the *ahpC* promoter compensate the loss of catalase peroxidase activity [60].
Rifampicin	*rpoB* (RNA polymerase β-subunit): mutations located in an 81 base pair "RIF resistance-determining region" [48]. Frequent mutations: codons 516, 526 and 531.
Pyrazinamide	*pncA* (pyrazinamidase/nicotinamidase): mutations in *pncA* disrupt the pyrazinamidase activity. *pnc*A mutations are highly diverse and scattered along the gene. *rpsA* (ribosomal protein S1): mutations in *rpsA* overexpress RpsA and restore *trans*-translation, essential for freeing scarce ribosomes in non-replicating stages [49].
Ethambutol	*embB* (arabinosyl transferase, part of the *embCAB* operon): mutations in *embB* restore arabinosyl transferase activity. Frequent mutation: codon 306.
Streptomycin	*rpsL* (S12 ribosomal protein): frequent mutations at codons 43 and 88. *rrs* (16S rRNA): frequent mutations are clustered in two regions, around nucleotides 530 and 915. *gidB* (16S rRNA 7-methylguanosine methyltransferase): mutations in *gidB* are associated with low-level STR resistance.
Kanamycin – Amikacin	*rrs* (16S rRNA): frequent mutations at nucleotides 1401, 1402 and 1484.
Capreomycin	*rrs* (16S rRNA): frequent mutations at nucleotides 1401, 1402 and 1484. *tlyA* (methyltransferase): mutations in *tlyA* modify each ribosomal subunits [52].
Fluoroquinolones	*gyrA* (A-subunit of gyrase): most *gyrA* mutations occur in a 320 base pair region. Frequent mutations: codons 74, 90, 91 and 94 [61]. *gyrB* (B-subunit of gyrase): most *gyrB* mutations occur in a 375 base pair region. Mutations in *gyrB* are rarer than in *gyrA*. Frequent mutation: codon 510.
Ethionamide	*ethA* (flavin adenosine dinucleotide): EthA oxidizes ETD into its active form. Mutations are distributed throughout the gene [62]. *inhA* gene and promoter (enoyl-acyl carrier protein reductase): mutations within *inhA* gene reduce InhA binding affinity. Mutations in the promoter upregulate target expression.

Although single nucleotide polymorphism (SNP) detection can be done by regular PCR amplification and sequencing, commercial kits are available and target particular genes (or gene regions), where the mutations of interest are located. These tools mainly focus on the identification of MDR-TB and have the advantage to be fast and sensitive enough to work on smear-positive sputum directly, thereby circumventing the need for lengthy *in vitro* growth culture. The simultaneous detection of *M. tuberculosis* infections and drug resistance allows to promptly treat the patient with adequate drugs without waiting for the DST outcomes under a potentially inefficient treatment. In 2009, the WHO approved the use of molecular line-probe assays the detection of drug resistance [55]. The best known of these assays is GenoType MTBDR*plus* (Hain Lifescience, Germany), which amplifies regions of *rpoB* and *katG* genes as well as the promoter of *inhA* in order to identify mutations associated with RIF and INH resistance. The visualization of the

outcome is done by colorimetric detection after hybridization of the labeled PCR products onto a strip coated with the corresponding oligonucleotides [63]. This assay is restricted in its application due to the required laboratory infrastructure (PCR technology) and its limited use among smear-negative TB cases [42]. Recently, the WHO endorsed the Xpert MTB/RIF assay (Cepheid, USA), which focuses on *rpoB* mutations, known to cause RIF resistance, indicating high risks of MDR-TB [42]. This fully automated assay showed a high sensitivity on sputum directly and promising results on smear-negative samples [40–42].

6. Genetic diversity

When TB started to re-emerge in the early 1990s, fuelled by the new-coming HIV epidemic, the global TB control relied on a 100 year-old diagnostic tool of poor sensitivity (smear microscopy), a largely ineffective 80 year-old vaccine (BCG), and decade-old drugs [64,65]. Facing this reality and the lack of alternative means to tackle the new-coming wave of TB, research programs were initiated to expand knowledge about the biology of *M. tuberculosis* and the MTBC genetic diversity.

M. tuberculosis is generally considered genetically monomorphic due to its low levels of genetic diversity, rare homoplasies (independent mutational events that result into an identical genotype among strains with a different ancestry) and virtual lack of homologous recombination [66]. However, in the last 20 years, research has shown that *M. tuberculosis* is more genetically variable than previously thought and that this strain-to-strain genetic variation matters [67,68]. The availability of the first molecular typing methods in the 1990s has shown that underlying differences in TB were not only due to environment or host factors, but also possibly related to the *M. tuberculosis* genetic background. The first whole *M. tuberculosis* genome sequence was released in 1998 [69] and paved the road for in depth investigations of the MTBC biology.

The MTBC comprises human-adapted species (*M. tuberculosis* and *M. africanum*) as well as animal-adapted species (*M. bovis*, *M. caprea*, *M. pinnipedii* and *M. microti*). All of them share 99.9% DNA homology [20]. For the purpose of understanding the phylogenetic structure of the MTBC, informative genomic polymorphisms have been identified. In 1997, before the first whole genome was published, Sreevastan *et al.* defined three *M. tuberculosis* genetic groups based on two SNPs in *gyrA* and *katG* [70]. Following the work from Sreevastan *et al.*, Brosch *et al.* showed that the deletion called TbD1 distinguished “modern” MTBC members and was absent in “ancestral”

members [71]. Based on several successive deletion events, they could propose an evolutionary pathway, notably indicating that *M. bovis* and *M. tuberculosis* shared a common ancestor. This refuted the long believed idea that humans acquired TB from animals during domestication and was further supported by the smaller size of the *M. bovis* genome compared to *M. tuberculosis* [71].

In the absence of horizontal transfer in MTBC, large sequence polymorphisms (LSPs) represent unique events and therefore robust markers for MTBC phylogenetic studies [72]. Using as reference the published whole genome sequence of the *M. tuberculosis* strain H37Rv, LSPs were identified in clinical strains. With this method, Hirsch *et al.* described *M. tuberculosis* clades associated with the patient's region of origin, indicating that in an urban setting (San Francisco in that study), hosts tended to contract *M. tuberculosis* of a genotype specific to the geographical region in which they were born [72]. Along these lines, Gagneux *et al.* identified LSPs in a comprehensive global strain collection and showed that human-adapted MTBC members are constituted of six phylogeographical lineages, each associated with preferential geographical regions and corresponding human populations [73]. In Africa, all six lineages were present, including the two West-African lineages (also referred to as *M. africanum*), which were not seen anywhere else, and the Indo-Oceanic lineage, the most ancestral of the six lineages. The close association between the host geographical origin and *M. tuberculosis* lineages reflects a longstanding co-evolution and suggests that *M. tuberculosis* spread out of East Africa together with human migration waves [73].

Methods based on DNA typing successfully contributed to the understanding of the genetic population structure of MTBC. Like LSPs, SNPs are phylogenetically informative markers, since the low genetic variability of *M. tuberculosis* makes mutations independent unique events. In 2008, Hershberg *et al.* used an extended multi-locus DNA typing approach and sequenced 1.5% of the *M. tuberculosis* genome in a global strain collection [7]. The resulting phylogeny was congruent with the one obtained from LSP-based analyses and confirmed the grouping of human-adapted MTBC members into six major lineages associated with geographical regions. Furthermore, Hershberg *et al.* showed that all animal-adapted MTBC members formed one in-group of the global phylogeny. Figure 4 depicts the phylogeny of MTBC and the global distribution of the six human-adapted MTBC lineages.

To explain the global spread of *M. tuberculosis*, an "out-of-and-back-to-Africa" scenario was proposed [7]. A first wave of migration out of Africa about 50,000 years ago brought humans and their infecting ancient *M. tuberculosis* strains (Lineage 1) to the Indian Ocean. In a subsequent wave, modern lineages (Lineages 2, 3 and 4) reached Eurasia, before further spreading to India and China.

Eventually, the contemporary history of migration brought back modern lineages to Africa through recent waves of travel, trade, and conquest [7]. Analyzing seven genes among MTBC strains and smooth tubercle bacilli (likely progenitors of MTBC), Guiterrez *et al*. demonstrated the longstanding co-evolution between tubercle bacilli and humans, suggesting that the last common ancestor of the MTBC could already have caused TB among early hominids in East Africa about 3 million years ago [5].

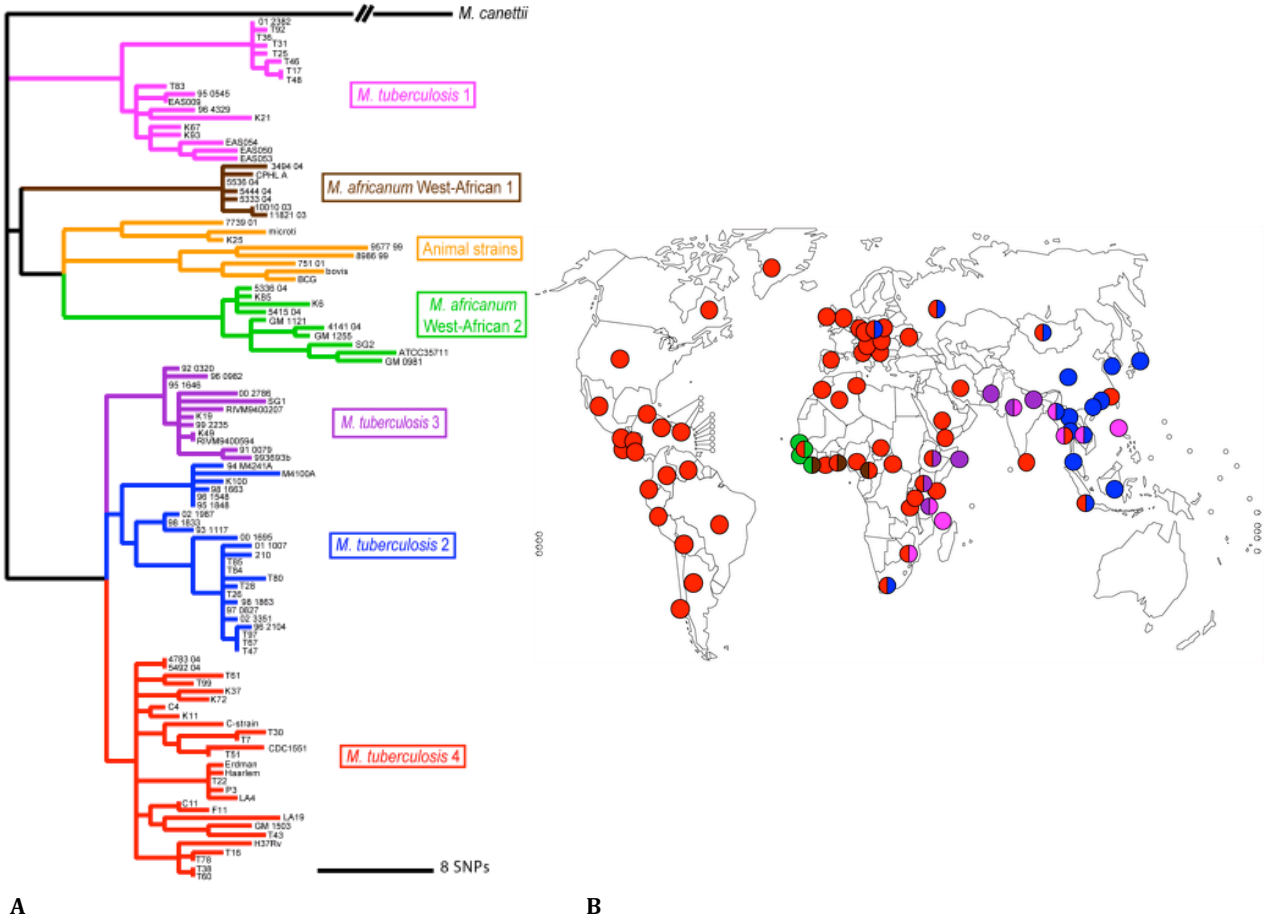

Figure 4: Panel A depicts the phylogeny of human- and animal-adapted MTBC members on the basis of SNPs identified by sequencing 89 genes in 108 MTBC strains. Panel B depicts the global distribution of the six main lineages of human-adapted MTBC members [7,65].

Recently, advances in next-generation sequencing technology contributed to lower its price. Whole genome sequencing is becoming a method of choice for the investigation of the MTBC genetic diversity, offering a complete picture of the tubercle bacillus genetics [65]. In 2010, Comas *et al.* sequenced 22 MTBC genomes and accurately confirmed the phylogeny previously obtained by LSPs or by multi-locus sequence analysis [74]. If the costs of next-generation sequencing continue

to decrease, whole genome comparisons could offer a robust way of investigating the genetic diversity of MTBC [75,76].

There is mounting evidence that the existing genetic differences between MTBC lineages have an impact on their phenotypes, and might consequently influence clinical outcomes. The phenotypic consequences of the MTBC genetic diversity have been reviewed in 2010 [76], and although many inconsistencies persist, evidence for strain-to-strain differences regarding virulence, immunogenicity and disease outcome could be shown. For example, ancient Lineage 6 strains (*M. africanum* West African 2) showed a slower progression to active TB compared to modern Lineage 2 strains (Beijing) in The Gambia, which illustrates the impact of genetic diversity at clinical level [77].

To comprehensively address the impact of MTBC genetic diversity in the future, a multidisciplinary systems epidemiology approach combining systems biology and epidemiology has been proposed in order to account for the interactions between pathogen, host and environmental factors [65,78].

7. Molecular epidemiology of tuberculosis

Molecular epidemiology is a powerful approach at the intersection between classical epidemiology and fundamental research, providing a better understanding of disease origins and of differences in pathogenesis, virulence, transmission or disease outcome. Molecular epidemiology is often applied in infectious disease research and allows epidemiologists to move beyond risk factors analyses and gain insights into the overall system of the disease [79]. Molecular epidemiology can be particularly useful to refine the understanding of TB outbreaks and for the general surveillance of the disease.

Genotyping (also referred to as “DNA fingerprinting”) is a key tool for molecular epidemiological investigations. In particular, genotyping allows to: i) distinguish a new infection (i.e. recent transmission) from a relapse (i.e. reactivation), ii) identify chains of transmission, iii) detect multiple co-infections, and iv) identify laboratory cross-contaminations. *M. tuberculosis* strains sharing the same genotype are considered as molecularly “clustered”. In population-based studies, molecularly clustered strains are assumed to be epidemiologically linked, while strains presenting a unique genotype (singletons) are assumed to result from a reactivated latent TB, acquired earlier

by the patient [66]. Using rates of clustered strains as a proxy for the estimation of recent TB transmission can provide useful information on the success of TB control programs.

M. tuberculosis genotypes can be obtained by various methods identifying specific markers. For molecular epidemiological purposes, it is important that the selected genetic markers are polymorphic enough to distinguish differences among unrelated strains, but yet stable enough to make the connection between strains that are indeed related [66]. In other words, genetic markers for molecular epidemiological investigations should allow discriminating truly epidemiologically related strains from unrelated strains as reliably as possible. The adequate genetic markers should be selected according to the purpose of the genotyping analysis and ideally, genetic markers for molecular epidemiology should not be used to infer phylogenies, which should rather rely on robust polymorphisms, namely with a low convergent evolution rate (see section 6) [68,80,81].

Since the early 1990s, several *M. tuberculosis* genotyping methods have been proposed in order to get a finer discrimination than what was previously obtained from classical biochemical assays. The ideal genotyping techniques for TB molecular epidemiology should be simple, rapid and affordable, while the resulting outcome should be easily comparable between laboratories [66]. The classical genotyping methods are insertion element (IS) restriction fragment length polymorphism (RFLP), spoligotyping and mycobacterial interspersed repetitive units-variable number of tandem repeats (MIRU-VNTR) (see below and table 3). Alternatively, SNPs and LSPs used for the investigation of MTBC population structure (see section 6) can also be informative in the frame of molecular epidemiological investigations. Furthermore, as the cost of high-throughput sequencing is decreasing, whole genome comparisons are becoming an attractive alternative, allowing the investigation of both molecular epidemiological and phylogenetic aspects.

IS6110 RFLP

IS*6110* RFLP was the first widely used method for *M. tuberculosis* genotyping and considered until recently as gold standard due to its high discrimination power [82]. IS*6110* is a 1361 base pair long IS specific to the MTBC. The discrimination is based on the number of IS*6110* copies inserted in the mycobaterial genome and the size of the DNA fragments in which they occur after enzyme restriction. This method developed in 1993 largely contributed to the first molecular epidemiological investigations of TB outbreaks. Despite being widely used, there are several drawbacks to this method. In particular, IS*6110* RFLP is labor intensive and requires large amounts of good quality DNA. It is therefore not applicable on sputum directly, but needs *in vitro* strain growth beforehand. Moreover, the discrimination power of this technique is not high enough for

strains with less than six IS*6110* insertions. An additional aspect making newer methods often preferred is the difficulty to reproduce and compare the obtained patterns between laboratories [66].

Spoligotyping

Another important *M. tuberculosis* genotyping method was developed in 1997 and was named spoligotyping for "spacer-oligotyping" [83]. Spoligotyping can add discrimination power when combined to IS*6110* RFLP. This method relies on polymorphisms in the clustered regularly interspaced short palindromic repeat (CRISPR) region of MTBC and offers an alternative to IS*6110* RFLP with the advantage of being easier to compare and to reproduce. The CRISPR region of the MTBC is called the Direct Repeat (DR) locus and is composed of identical 36 base pair repeats separated by 94 unique "spacers" of 35-41 base pairs. Although the order of the spacers is conserved, some get lost and the presence or absence of 43 selected spacers allows discriminating the strains. After PCR amplification of the spacers, the amplicons are hybridized onto a membrane spotted with the corresponding oligonucleotides. The resulting pattern of present or absent spacers revealed by chemiluminescence can be digitalized for identification on the global spoligotype database (SpolDB4) [84]. Although more convenient than IS*6110* RFLP, spoligotyping is less discriminative and prone to convergent evolution (when identical spoligotypes occur in unrelated strains as a result of homoplasy) [81]. Furthermore, this method can unfortunately not discriminate variations among Beijing strains, which all share the same spoligotype pattern.

MIRU-VNTR

The third widely used method for *M. tuberculosis* genotyping called MIRU-VNTR was developed in 2000 and is often considered as the new gold standard replacing IS*6110* RFLP [85–87]. The polymorphism is based on the variable number of tandem repeats at distinct loci in the genome. The discrimination power of MIRU-VNTR largely depends on how many loci are assessed. Protocols based 12, 15 or 24 loci have been proposed, with the highest discrimination obtained when the 24 locus set is used [66]. After PCR amplification of the loci, the determination of repeat numbers can be performed by visualization on agarose gels. For a higher throughput, a capillary sequencer can be used to measure the size of each amplicon [86]. The result is a numerical value composed of the number of repeats observed at each locus. This value can be matched to an online database for identification and comparison [88]. Despite the good discrimination power, the rapidity of the protocol (with capillary sequencer) and the high reproducibility, MIRU-VNTR can be prone to homoplasy, like spoligotyping [81]. Consequently, similar DNA fingerprints obtained from those

methods can occur in unrelated strains as a result of convergent evolution and therefore, they should not be used for phylogenies.

Table 4: List of the classical methods used for the genotyping of *M. tuberculosis*. Adapted from [66].

Genotyping method	Principle	Technical requirements	Advantages	Disadvantages
IS*6110*-RFLP	Based on the variability in IS*6110* number of copies and molecular weights of DNA fragments in which the IS are found.	• PCR amplification and probe labeling • 2–3 µg of pure DNA • Southern blotting • Visual or software-based comparison	• High discriminatory power • Widely used	• Limited discriminatory power in strains with ≤ 5 IS*6110* bands • Lengthy process • Difficult to compare results between laboratories
Spoligotyping	Based on the presence or absence of spacers in the DR locus.	• PCR amplification • Specific blotter and membrane	• PCR-based • Data in exchangeable format • Highly reproducible	• Limited discriminatory power
MIRU-VNTR	Based on polymorphisms in up to 24 MIRU-VNTR loci.	• PCR amplification • Gel electrophoresis or sequencer	• PCR-based • High discriminatory power • Data in exchangeable format	• Set of 12 loci less discriminatory than IS*6110* RFLP • Determination of band size less reproducible with electrophoresis than with sequencer • High cost of sequencer method

Deletions and SNP typing

Highly discriminatory methods such as IS*6110* RFLP, spoligotyping or MIRU-VNTR are particularly useful in clinical settings, when circulating strains need to be tracked and distinguished from each other with a high resolution. Phylogenetically informative polymorphisms (see section 6) such as SNPs and LSPs provide an unambiguous classification of clinical strains, which can also be useful in the frame of molecular epidemiological investigations. In addition, because these makers are unique and likely irreversible events, they are less prone to convergent evolution, and thereby adequate for a robust phylogenetic classification of strains [68]. Comas *et al.* showed that phylogenies based on LSPs and DNA sequences were highly congruent and generated negligible homoplasy [81]. An illustration of convergent evolution in the DR locus of MTBC was given by the recent report by Fenner *et al.*, showing that strains sharing the classical "Beijing" spoligotyping pattern can in fact belong to different lineages, as identified by specific SNPs and genomic deletions [89].

Following the advances in understanding the MTBC population structure by SNPs or LSPs analyses, various classification have successively been proposed. As already mentioned in section 6, Sreevastan *et al.* described in 1997 the distinction of three groups among MTBC members (referred to as "principal genetic groups") based on the presence or absence of SNPs at position 463 in *katG*

and 95 in *gyrA* [70]. Following the release of the H37Rv genome sequence in 1998 [69], studies from Gordon *et al.* and from Behr *et al.* described the presence of genomic deletions in *M. bovis* BCG strains (referred to as "region of difference"), which were absent in *M. tuberculosis* [90,91]. In 2002, Brosch *et al.* proposed a new scenario for the evolution of MTBC based on the successive accumulation of regions of difference [71]. While TbD1 classifies MTBC members into "modern" or "ancestral" strains (see section 6), further successive loss of DNA stretches were found to more specifically characterize *M. africanum*, *M. bovis* or *M. bovis* BCG [71]. In 2004, Baker *et al.* identified four *M. tuberculosis* groups by multilocus sequence typing on housekeeping genes [92]. Also in 2004, Tsolaki *et al.* identified 68 LSPs (similarly called "region of difference") by DNA microarray, which were absent in some *M. tuberculosis* clinical strains when compared to H37Rv [93]. In 2006, Gutacker *et al.* proposed a subdivision into nine groups (referred to as "major genetic clusters") defined by a subset of specific SNPs [94], while Filliol *et al.* used SNPs to define six phylogenetically distinct groups associated with the geographical origin of patients [95]. Still in 2006, Gagneux *et al.* screened a large global collection of *M. tuberculosis* strains with LSPs following the work of Tsolaki *et al.* [93], and showed that *M. tuberculosis* comprised six main lineages (two of them referring to *M. africanum*) associated with the host's country of origin [73].

Thank to the comprehensiveness of complete genome sequences, information can be obtained at both epidemiological and phylogenetic levels. In 2010, whole genome sequencing was used on 22 MTBC strains [74] and showed a phylogenetic structure congruent with the classification in six lineages proposed by Gagneux *et al.* in 2006 [73]. Nevertheless, before whole genome sequencing becomes more readily available, a dual genotyping approach is recommendable, whereby highly discriminatory typing methods such as IS*6110* RFLP, spoligotyping or MIRU-VNTR may be used for strain characterization, while more robust markers such as SNPs or LSPs would be preferred for the unambiguous assignment of strain lineages [81].

Project description

Burden of tuberculosis in Papua New Guinea

Papua New Guinea (PNG) is the largest country in the Pacific after Australia, occupying the eastern half of the island of New Guinea (the second largest island in the world after Greenland) and other smaller islands. The mainland accounts for about 85% of the overall country surface and about 600 smaller islands constitute the remaining 15%. The capital city is Port Moresby and the country is divided into 20 provinces, with a total population of 7 million inhabitants in 2010 [96,97]. PNG belongs to the region called Melanesia, located in the northeast of Australia and notably comprising the Solomon Islands, Vanuatu, New Caledonia, Fiji and PNG.

The country presents a challenging environment for all health programs, especially because of its rough topology and very low population density, often scattered in small and isolated communities. Only about 12% of the population lives in urban areas and very few roads are paved. Therefore, many journeys need to be done on foot or by airplane, although the prices of domestic flights are prohibitive for most Papua New Guineans [96,98]. Government and churches provide nearly 100% of the health care services in PNG. In fact, churches alone operate 46% of the health facilities, mostly in the rural areas [96].

In addition to the difficult topological aspects, the implementation of TB control in PNG is further impaired by the limited availability of resources and appropriately trained staff [96]. The first countrywide TB control activities were initiated in the 1950s and short course chemotherapy was introduced in 1987 [99]. The national government decided to launch the DOTS strategy in 1997 in a phased manner [99]. Despite these actions, the control plan was seriously criticized at the end of the 1990s, when Levy *et al.* characterized the PNG TB control program by its poor diagnosis rates, high levels of treatment defaulting and low cure rates [100]. The Global Fund reported that the quality of TB diagnosis and treatment in the DOTS-covered districts was unacceptably low and therefore started to support the strengthening of the PNG TB program in 2006, with the goal of halving the TB prevalence and death rates by 2020 (compared to the 1990 levels) [99]. Nevertheless, in 2011, only some provinces had implemented the DOTS strategy and still fairly scarce information was available about the current TB epidemic in PNG.

The WHO estimates indicate that PNG belongs to the seven countries with the highest TB burden in the Western Pacific region (together with Cambodia, China, Lao People's Democratic Republic, Mongolia, the Philippines and Viet Nam) [96]. The PNG TB program reports that TB is the second most common reason for hospital bed occupancy and the forth commonest cause of death during hospital stays [101]. The WHO estimated that in 2010, the incidence rate of TB in PNG reached 21,000 new cases (equivalent to 303 new TB cases per 100,000 population in a year), while the TB prevalence was of 465 cases per 100,000. In addition, 2,900 people died of TB in 2010 [97].

In 2007, six of the seven high TB burden countries in the Western Pacific region had a treatment success rate above the Global Stop TB target of 85% among new pulmonary smear-positive cases, except PNG with only 39% treatment success (although treatment outcome was not available for all cases) [96]. In 2009, the treatment success rate had increased to 58%, but was yet still below the 85% target [12].

Data about the burden of drug-resistant TB in PNG are limited as well, principally owing to the current absence of adequate infrastructures to perform DST in the country. In the meantime, a few health centers in PNG initiated a partnership with the Queensland Mycobacterium Reference Laboratory (QMRL) in Brisbane, Australia, where samples can be sent to for DST. This laboratory also conducted studies in the Torres Strait Islands, which are part of the open border region separating the north of Australia from the south of the PNG Western Province. Gilpin *et al.* reported that out of 60 patients diagnosed with TB between 2000 and 2006 in that region, 25.0% had MDR-TB associated with high mortality rates [102]. In 2011, Simpson *et al.* confirmed the high rate of MDR-TB in that region [103]. In contrast, the latest estimations published by the WHO predicted 5% of MDR-TB among new TB cases in PNG, and 24% among re-treatment cases in 2010 [97]. XDR-TB has never been reported in PNG.

In the Western Pacific region, the prevalence of HIV in incident TB cases was estimated to be the highest in Cambodia (15%) followed by Malaysia (12%) and PNG (3.8%) [96]. However, HIV testing is not yet routinely performed among TB suspected individuals in PNG: WHO reported that only about 7% of TB patients have a known HIV status [97].

Study rationale

There is a great need for more data about the current TB epidemic in PNG. TB and more particularly drug-resistant TB as well as HIV-associated TB are major public health issues worldwide and only scarce data describing the current situation in PNG is available. Increasing the understanding of the TB characteristics in PNG can provide the necessary baseline information to better address the control of this disease.

Identifying drug-resistant MTBC strains circulating in the population is important in order to adapt therapies as early as possible and thereby to decrease the risks of treatment failure or fatal outcomes. Moreover, the adequate management of drug-resistant TB is necessary to reduce the spread of already resistant strains in the community and to interrupt the resulting iterative circle fuelling the burden of TB. Indeed, if TB continues to be treated without an adequate monitoring of drug susceptibility, resistance may develop unnoticed under ongoing therapy and amplify the pool of circulating drug-resistant strains.

Apart from the lack of information regarding drug resistance, PNG remains a fairly white spot on the map of MTBC genetic diversity and data about the molecular background of strains circulating in this region is missing. Such information is important since the genetic variations among MTBC members have been shown to impact on various aspects of the disease outcome, such as immunogenicity, response to treatment or propensity to develop into active TB [76,78].

Study site

PNG has fascinated many researchers, especially anthropologists and linguists attracted by the great socio-cultural and linguistic diversity of Papua New Guineans. This diversity is particularly high among isolated communities living in remote areas and which maintained limited contacts with the outside world during centuries [104].

The PNG Institute of Medical Research (PNG IMR) was established in 1968 with the primary goal of conducting research on the health problems of Papua New Guineans. The PNG IMR is a government-funded research institution, one of the oldest independent national research institutes

in the developing world. All findings obtained by the PNG IMR are systematically communicated to the PNG Ministry of Health for a prompt implementation. The headquarters of the PNG IMR are located in Goroka, the capital of the Eastern Highlands Province and other branches are located in Madang, Maprik, Port Moresby and Wewak. The Swiss Tropical and Public Health Institute (Swiss TPH) began to collaborate with the PNG IMR approximately 20 years ago. In 2008, the PNG IMR and the Swiss TPH initiated a research agenda on TB, starting with the present project.

This study was based in Madang, the capital of the Madang Province situated on the north coast of PNG. The main hospital of the province, called Modilon General Hospital is located in the center of Madang town and comprises a TB clinic. Next to the hospital, laboratory facilities were built in 2007-2008 and since 2009, also host a unit dedicated to TB investigations. This TB laboratory has one sterile hood and can therefore be used for processing sputum according to state of the art protocols. In addition, regular light microscopy as well as fluorescent microscopy is available for the screening of sputum smears. Growing *M. tuberculosis* from sputum is however not allowed since the laboratory is built as biosafety level 2, and not according to the highly restrictive biosafety level 3 rules. For that reason, DST is done at the QMRL in Australia in the frame of a collaborative partnership.

Research partnerships

Beside the core partnership between the PNG IMR and the Swiss TPH, this study was conducted in collaboration with the following institutions:

Queensland Mycobacterium Reference Laboratory, Brisbane, Australia: Because of the absence of biosafety level 3 laboratories in PNG, *in vitro* growth and phenotypic DST were conducted by the QMRL under the direction of Robyn Carter and Dr. Christopher Coulter. DST outcomes were then reported back to the TB clinic in Madang electronically, so that patient regimens could be adapted accordingly.

Institute of Medical Microbiology, University of Zurich, Switzerland: Before the opening of a biosafety level 3 laboratory in 2010 and the establishment of routine *M. tuberculosis* genotyping at the Swiss TPH, a comprehensive training on general mycobacteriology and spoligotyping was provided by the Institute of Medical Microbiology under the direction of Prof. Erik C. Böttger.

Research Center Borstel, Germany: An intensive training on genotyping methods as well as MIRU-VNTR strain typing was done in collaboration with the Research Center Borstel under the direction of Dr. Stefan Niemann.

Aims and objectives

Within the proposed project, we aimed at supporting the PNG TB control program, which requested the PNG IMR to initiate investigations on TB. The longstanding collaboration between the PNG IMR and the Swiss TPH perfectly fitted into this framework with the aim of generating data on the molecular epidemiology of TB and introducing some useful and missing techniques in PNG for a sustainable monitoring of TB in the country. This project was planned on the basis of a bilateral collaboration between the Papua New Guinean and Swiss counterparts.

Aims

The overall aim of this study was to provide insights into the characteristics of *M. tuberculosis* strains isolated from pulmonary TB patients visiting the TB clinic of Modilon General Hospital in Madang as well as two additional health centers in the close vicinity (Mugil and Sek). The project focused on two main aspects: i) assessing the burden of drug-resistant TB, and ii) exploring the genetic diversity of *M. tuberculosis* in this region. All strains for this project were collected first during a pilot study conducted in 2005-2007 and then in the frame of an ongoing treatment cohort study initiated in April 2009.

Furthermore, in parallel to the research aspects mentioned above, the project comprised a capacity building component aiming at setting up basic bacteriology techniques in the newly built laboratory located next to the TB clinic, in the compound of the Modilon General Hospital. In the frame of two field visits in 2009 and 2010, Papua New Guineans research fellows were trained on general laboratory rules related to TB work and more specifically on sputum manipulation, encompassing sputum decontamination, inoculation of growth medium for culture, and appropriate sample storage. This component aimed at sustainably improving laboratory capacities and research-oriented skills in the field of TB, thereby contributing to make the Papua New Guinean counterparts independent in the daily manipulation of patient TB samples.

This project hopefully established solid bases for long term countrywide TB investigations in order to gain the necessary knowledge for an adequate control of the disease in PNG.

Objectives

Objective 1: To assess the drug resistance profile of the *M. tuberculosis* strains by the mean of phenotypic DST for the most important first-line and second-line anti-TB drugs.

Objective 2: To identify mutations associated with drug resistance by sequencing *M. tuberculosis* genes known to harbor resistance-conferring mutations.

Objective 3: To investigate the genetic diversity of *M. tuberculosis* strains using various genotyping methods generating different levels of strain-to-strain discrimination.

Objective 4: To identify the potential patient risk factors associated with drug resistance and with particular *M. tuberculosis* genetic backgrounds.

Objective 5: To evaluate the importance of *M. tuberculosis* transmission based on the identification of molecular strain clusters.

Chapter 1

Genetic diversity of *Mycobacterium tuberculosis* in Madang, Papua New Guinea

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Summary

Setting

Madang and surroundings, Papua New Guinea (PNG)

Objective

To characterize the genetic diversity and drug susceptibility of *Mycobacterium tuberculosis* isolates collected in Madang and surroundings.

Design

M. tuberculosis was isolated in sputum samples from active pulmonary tuberculosis cases. Drug resistance profiles were obtained by drug susceptibility testing. *M. tuberculosis* lineages were identified by single nucleotide polymorphisms and sub-typing was performed by spoligotyping. Spoligotyping and 24 locus mycobacterial interspersed repetitive units variable number of tandem repeats were combined to identify molecularly clustered isolates.

Results

The 173 *M. tuberculosis* isolates collected belonged predominantly to the Euro-American lineage (Lineage 4) and the East-Asian lineage (Lineage 2). Multidrug-resistant *M. tuberculosis* were observed in 5.2% isolates. Lineage 2 *M. tuberculosis* (includes "Beijing" genotype) were significantly associated with any drug resistance (Odds Ratio: 5.2, 95% Confidence Interval 1.8–15.1). Cluster analyses showed 44.4% molecularly clustered isolates, suggesting transmission of *M. tuberculosis* in the community, including transmission of primary drug-resistant *M. tuberculosis*.

Conclusion

This data provides the first insight into the molecular characteristics of *M. tuberculosis* in the Madang area of PNG and indicates substantial drug resistance with evidence for ongoing transmission.

Introduction

Tuberculosis (TB) in Papua New Guinea (PNG) is a concern with an estimated incidence > 10 times higher than in other Pacific island countries (1,2). In 2010, the World Health Organization (WHO) estimated that the yearly TB incidence in PNG was 303 per 100,000 population, and the prevalence of multidrug-resistant (MDR) TB in new cases was estimated at 5% (3). However, because of the limited access to health care and poor record keeping in rural PNG, those numbers are likely underestimated.

Previous publications on the PNG Western Province showed 25.0% MDR-TB among patients seeking care in Australian territories across the border (4,5). Unfortunately, facilities for *M. tuberculosis* culture and drug susceptibility testing (DST) are lacking in PNG. However, a collaborative agreement with the Queensland Mycobacterial Reference Laboratory (in Australia) allows samples collected in the Madang region to be sent to Australia for culture and DST.

M. tuberculosis harbors higher genetic diversity than previously thought (6,7). On the basis of phylogenetically informative markers such as large sequence polymorphisms or single nucleotide polymorphisms (SNPs), six phylogenetic "lineages" of *M. tuberculosis* have been defined (6–9). Each of these lineages is closely associated with specific geographical regions, and preferentially infects individuals originating from these regions (6,9). Evidence that *M. tuberculosis* genotypes might influence disease development is increasing (see (10) for review); in particular, the association between Beijing strains (Lineage 2) and drug resistance has often been reported (11,12). Within SNP-defined lineages, further genotypic resolution can be obtained by spoligotyping (13). Spoligotyping combined with mycobacterial interspersed repetitive units variable number of tandem repeats (MIRU-VNTR) (14) can be used to define clusters of strains sharing identical molecular profiles, and thus indicating recent transmission (15,16).

To date, the diversity of *M. tuberculosis* in PNG remains largely unexplored. Such data is necessary to better understand underlying differences in *M. tuberculosis* infections and assess *M. tuberculosis* transmission dynamics in the region. The present study provides the first data on drug resistance and genetic diversity of *M. tuberculosis* strains collected in Madang and surroundings.

Material and methods

Study setting and patient characteristics

Madang is located on the North coast of PNG with a population of about 27,000 inhabitants in 2005 (365,000 inhabitants in Madang Province). In 2005-2007, a pilot study was conducted at the Modilon General Hospital, followed by a treatment cohort study initiated in April 2009 at the same hospital plus two rural health centers located nearby. The Modilon General Hospital is the main hospital of Madang Province. All microscopically confirmed pulmonary TB patients aged $\geq$ 15 years coming for TB consultation were eligible or when other clinical evidence suggested smear-negative TB. Patient treatment and follow-up procedures were carried out according to the national TB program, which in Madang Province includes the directly observed treatment, short-course (DOTS) strategy. Demographic and clinical data were prospectively collected since the initiation of the treatment cohort study in April 2009. Samples from the pilot study were included in the molecular analyses; however, no corresponding patient demographic or clinical data were available for these.

HIV status was assessed by Determine® HIV-1/2 (Inverness Medical Innovations, Australia) and confirmed by ImmunoComb® HIV 1&2 TriSpot Ag-Ab (Orgenics, Israel).

Sample collection and processing

In the TB laboratory of Madang, all sputum samples collected from pulmonary TB patients were examined by light and fluorescent microscopy. Samples were decontaminated according to Petroff's method (17). Centrifuged pellets were transferred into MGIT (Mycobacterial Growth Indicator Tubes – Beckton Dickinson, USA). Inoculated MGIT were transported to the Queensland Mycobacterium Reference Laboratory in Australia for culture and DST. DNA was extracted from cultured strains with InstaGene Matrix (Bio-Rad, USA) and sent to the Swiss Tropical and Public Health Institute for molecular analyses.

Genotyping

M. tuberculosis lineages were identified by real-time PCR targeting lineage-specific SNPs (Applied Biosystems, USA) as described previously (6,7,18,19).

Sub-lineage differences were identified by spoligotyping (13). Specific shared international type (SIT) and spoligotype families were obtained from SpolDB4 (www.pasteur-guadeloupe.fr:8081/SITVITDemo/index.jsp, (20)). Spoligotypes not reported in SpolDB4 database were designated as orphans.

MIRU-VNTR at 24 loci was performed as previously described (14) in collaboration with the Research Center Borstel in Germany. Clusters were defined as strains sharing identical spoligotype and MIRU-VNTR patterns and used as a proxy to evaluate *M. tuberculosis* transmission. MIRU-VNTR*plus* (www.miru-vntrplus.org, (21)) was used to match spoligotypes and MIRU-VNTR patterns. Incomplete MIRU-VNTR patterns were excluded from the cluster analysis.

Drug resistance

DST was performed by the proportion method (22) using the BACTEC MGIT 960 system (Beckton Dickinson, USA) with the following drug concentrations: rifampin (1.0 µg/mL), isoniazid (0.1 and 0.4 µg/mL), ethambutol (5.0 µg/mL), pyrazinamide (100 µg/mL), streptomycin (1.0 µg/mL), amikacin (1.0 µg/mL), kanamycin (5.0 µg/mL), ofloxacin (2.0 µg/mL), capreomycin (2.5 µg/mL), ethionamide (5.0 µg/mL), *p*-aminosalicylic acid (4.0 µg/mL), and cycloserine (50.0 µg/mL). Isolates resistant to one drug were considered monoresistant, isolates resistant to at least INH and RIF were considered MDR and isolates resistant to at least one drug but not MDR were considered polyresistant.

Statistical analyses

Chi^2 or Fisher's exact tests were used to assess differences in categorical variables and Mann-Whitney U tests for continuous variables. Odds ratios were obtained from univariate logistic regressions. Statistical analyses were done with Stata 10.1 (StataCorp, USA).

Ethical approval

The PNG Institute for Medical Research Review Board, and the PNG National Medical Research Advisory Council's Ethics Committee approved the study protocol. The Ethikkommission beider Basel in Switzerland was informed about the study. Written informed consent was obtained from all patients enrolled in the study.

Results

Patient characteristics and sample set

In a pilot study, 60 patients with pulmonary TB were enrolled in 2005-2007. From April 2009 to October 2010, 201 patients were recruited into a treatment cohort study, during which detailed demographic and clinical data were collected. Table 1 depicts the patient characteristics at enrollment.

All DST and molecular analyses were performed on the 60 isolates from the pilot study plus the 118 from the 2009-2010 treatment cohort study. Five (2.8%) samples were excluded because of mixed infections as indicated by the presence of two alleles at > 1/24 MIRU-VNTR loci. Hence, a total of 173 isolates were included for subsequent analyses.

Strain genotypes

SNP-based lineage typing showed that 133/173 (76.9%) isolates belonged to the Euro-American lineage (Lineage 4), 39 (22.5%) to the East-Asian lineage (Lineage 2, includes Beijing genotype), and 1 (0.6%) to the Indo-Oceanic lineage (Lineage 1). No patient characteristic was significantly associated with Lineage 2 as opposed to Lineage 4 *M. tuberculosis* (table 2).

Within lineages, isolates were sub-divided by spoligotyping. Among the 173 strains spoligotyped, 34 different patterns were observed, with 19 (11.0%) isolates belonging to 14 different orphan spoligotypes (see table 3 for the list of spoligotypes observed). The three most prevalent spoligotypes were SIT 393 (39/173, 22.5%), SIT 1 (38/173, 22.0%) and SIT 53 (26/173, 15.0%). Within Lineage 4, the most frequent spoligotype families were T1 (87/133, 65.4%), LAM (11/133, 8.3%) and X1 (10/133, 7.5%). Orphans within Lineage 4 accounted for 18/133 (13.5%). Within Lineage 2, the main spoligotype family was SIT 1, i.e. the Beijing genotype (38/39, 97.4%); one orphan spoligotype was found in this lineage (2.6%). The single Lineage 1 isolate (Indo-Oceanic lineage) belonged to the EAI family.

The lineage distribution among samples from the 2005-2007 pilot study and among samples collected in 2009-2010 did not differ significantly. Regarding the spoligotype prevalence, SIT 1 and SIT 119 were more frequent in 2009-2010 than in 2005-2007 (SIT 1: 24.8% versus 16.7% and SIT 119: 8.9% versus 0%), whereas SIT 102, SIT 42 and SIT 53 were less frequent in 2009-2010 than in 2005-2007 (SIT 102: 7.1% versus 13.3%, SIT 42: 4.4% versus 8.3%, and SIT 53: 10.6% versus 23.3%).

Drug resistance

DST was successfully conducted for 172/173 of the isolates. Among these, 27 (15.7%) were resistant to at least one drug, and the remaining 84.3% were pan-susceptible. Table 4 shows the different combinations of drug resistance. Isoniazid monoresistance was seen in 9 (5.2%) patients and rifampin monoresistance was seen in 1 (0.6%) patient. MDR-TB occurred in 9 (5.2%) patients, no extensively-resistant TB was observed. Neither prevalence of resistance to at least one drug nor prevalence of MDR-TB significantly differed among samples from the 2005-2007 pilot study and from the 2009-2010 treatment cohort study.

Univariate logistic regressions were performed to estimate risk factors associated with drug-resistant *M. tuberculosis* among patients from the 2009-2010 study for whom characteristics were available (table 5). Lineage 2 isolates (East-Asian lineage) showed a statistically significant association with resistance to any drug (OR: 5.2, 95% CI 1.8–15.1).

Transmission clusters

MIRU-VNTR typing at 24 loci was complete for 162/173 (93.6%) isolates and was used together with spoligotyping to define molecularly clustered *M. tuberculosis* isolates, indicating recent transmission. Out of 162 isolates genotyped with both methods, 72 (44.4%) were in molecular clusters. In total, 24 different clusters comprising 2 to 7 isolates were observed (median: 2 isolates/cluster) and 6 (25.0%) clusters involved drug-resistant isolates. The remaining 18 clusters involved pan-susceptible isolates only. The proportion of drug resistance was not significantly different in clustered (12/72, 16.7%) versus non-clustered (13/90, 14.4%) isolates. Similarly, MDR isolates were equally distributed between clustered (4/72, 4.4%) and non-clustered isolates (4/90, 5.6%). Table 6 describes risk factors associated with *M. tuberculosis* strain clustering. The proportion of clusters did not significantly differ between patients from the 2005-2007 pilot study and patients recruited in 2009-2010 (36.7% and 49.0%, respectively).

Discussion

Little is known about TB in PNG. In particular, data on drug resistance are scarce, and PNG remains a white spot on the map of *M. tuberculosis* phylogeographic diversity (7). In the present report, we investigated *M. tuberculosis* isolates from pulmonary TB patients in Madang and surroundings. DST showed that 15.7% of the strains were resistant to at least one drug and 5.2% strains were MDR. Strains collected in this region predominantly belonged to Lineages 4 (Euro-American lineage) and 2 (East-Asian lineage).

The proportion of MDR-TB strains reported here was lower than the 25.0% previously observed in the Western Province (4,5), although there, a bias towards sicker individuals seeking treatment in the nearby Australian territories cannot be excluded. In the absence of DST facilities in the country, no national surveillance data are available for comparison. Apart from drug resistance, HIV is another factor impacting on the TB burden, more particularly in Africa (23,24). In PNG, WHO estimated an HIV prevalence in the general population of 0.9% in 2009, and among TB patients, 3.8% were HIV co-infected, although most TB patients are not tested for HIV (1,25). In this study, HIV testing was routinely performed and 12/201 (6.9%) TB patients were HIV positive.

A great human genetic diversity can be found in PNG, and more generally in Melanesia, partly because of the country topology and the remoteness of many communities, especially in the islands' interiors (26). Considering that *M. tuberculosis* appears to have co-evolved with human populations for millennia (6), particular or novel *M. tuberculosis* lineages could be expected in PNG. However, in the sample set reported here, mainly Lineage 4 and Lineage 2 were observed. The large proportion of Lineage 4 *M. tuberculosis* strains suggests that the appearance of TB in PNG may be associated with the arrival of Europeans and Australians in the past centuries. *M. tuberculosis* genotypic data from other regions in the country would provide useful information to better understand the emergence of TB in PNG. It would be interesting to see whether the same *M. tuberculosis* genotypes are found in highland versus coastal regions of PNG. Human genetic differences between these communities were identified by mitochondrial DNA analyses, illustrating the more ancient origin of highland populations (27). This difference could possibly also be reflected in the infecting *M. tuberculosis* populations.

Comparing the genetic diversity of *M. tuberculosis* in PNG with observations from surrounding countries would be highly informative, but unfortunately, we know of no such data in any other Melanesian country. Only Gilpin *et al.* reported the genotypes of MDR strains collected in the PNG

Western Province (4). Using 17-locus MIRU-VNTR and IS*6110* restriction fragment length polymorphism, they showed that all MDR strains belonged to the Beijing genotype.

The proportion of orphan spoligotypes (11.0%) also revealed that not all the *M. tuberculosis* genotypes observed in the Madang region were reported before, and it would be interesting to see whether these fingerprints are observed in other regions of PNG, or in countries nearby.

We observed that 38.5% of Lineage 2 strains (East-Asian lineage) were resistant to at least one drug, in contrast with 9.1% among Lineage 4 strains (Euro-American lineage). The association of Beijing strains with drug resistance has been reported repeatedly (12,28,29). Furthermore, Beijing strains were more frequently observed in younger patients in Vietnam (30), but in the present study, no age difference was observed.

The potential effect of mixed *M. tuberculosis* infections has not often been considered in epidemiological studies. Moreover, being able to detect this phenomenon depends on the genotyping methods (31,32). If not especially looked for, mixed *M. tuberculosis* infections often remain undetected. In this study, five (2.8%) multiple infections were defined on the basis of mixed MIRU-VNTR patterns at $\geq$ 2 loci, as previously proposed (15). Mixed signals at only one MIRU-VNTR locus (3/178 cases) were allowed as potential within host strain diversity.

Molecular clusters can be used as a proxy to estimate recent *M. tuberculosis* transmission. In this setting, transmission seemed high, with 44.4% strains in clusters. Although little information is available about transmission in surrounding countries, frequent transmission was also observed in Taiwan with 61.6% clustered strains (33). However, the interpretation of *M. tuberculosis* transmission based on molecular clusters should be taken with caution. On the one hand, transmission may be overestimated knowing that spoligotyping cannot discriminate variation among Beijing strains and that the discrimination power of 24-locus MIRU-VNTR for Lineage 2 sub-typing has also been questioned (18). Moreover, potential chains of transmission should ideally be validated by contact tracing to confirm true epidemiological links. Such data were not available in our study and would be difficult to collect retrospectively. On the other hand, it is possible that transmission was underestimated, since only a proportion of TB patients in Madang region were recruited into this study, and over a limited time period. Consequently, some genotypes observed as unique may in fact also have resulted from recent transmission, although other patients infected with the same strain were not represented in the study population.

In summary, this report provides baseline information about *M. tuberculosis* strains circulating in the Madang region of PNG. Despite the limited number of patients included, the *M. tuberculosis* isolates analyzed indicate substantial drug resistance, and suggest that TB in this region was at least in part introduced by European and Asian immigrants. Furthermore, the cluster analyses indicate ongoing *M. tuberculosis* transmission, including transmission of drug-resistant strains. There is an urgent need to establish capacity for diagnosing and treating drug-resistant TB in PNG.

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Tables

Table 1

Socio-demographic, clinical and laboratory characteristics of patients recruited between April 2009 and October 2010 (n=201).

Characteristic	Subcategory	All (n=201)	DNA sample available (n=118, 58.7%)	DNA sample not available (n=83, 41.3%)
Sex, n (%)	Men	102 (50.8)	68 (57.6)	34 (41.0)
	Women	99 (49.2)	50 (42.4)	49 (59.0)
Age – median years (IQR)		28 (21-37)	26 (20.5-32.5)	32 (22-45)
Age groups – years, n (%)	15-24	70 (34.9)	48 (40.7)	22 (26.5)
	25-34	67 (33.3)	43 (36.4)	24 (28.9)
	35-44	28 (13.9)	15 (12.7)	13 (15.7)
	>45	34 (16.9)	10 (8.5)	24 (28.9)
	Unknown	2 (1.0)	2 (1.7)	0 (0)
Province of origin, n (%)	Madang Province	86 (42.8)	48 (40.7)	38 (45.8)
	East Sepik Province	49 (24.4)	29 (24.6)	20 (24.1)
	Other provinces	42 (20.9)	23 (19.5)	19 (22.9)
	Unknown	24 (11.9)	18 (15.2)	6 (7.2)
Education, n (%)s	No education	36 (17.9)	15 (12.7)	21 (25.3)
	Primary education	110 (54.7)	73 (61.9)	37 (44.6)
	Higher education	55 (27.4)	30 (25.4)	25 (30.1)
HIV, n (%)	Positive	12 (6.0)	5 (4.2)	7 (8.4)
	Negative	162 (80.6)	97 (82.2)	65 (78.3)
	Unknown	27 (13.4)	16 (13.6)	11 (13.3)
BMI – median (IQR)		18.5 (16.3-21.1)	17.4 (15.9-21.4)	19.6 (16.8-21.4)
Smoking, n (%)	Yes	88 (43.8)	56 (47.5)	32 (38.5)
	No	113 (56.2)	62 (52.5)	51 (61.5)
Sputum smear result, n (%)	Negative	13 (6.5)	5 (4.2)	8 (9.6)
	Scanty	34 (16.9)	10 (8.5)	24 (28.9)
	1+	32 (15.9)	11 (9.3)	21 (25.3)
	2+	16 (8.0)	15 (12.7)	1 (1.2)
	3+	103 (51.2)	76 (64.4)	27 (32.6)
	Unknown	3 (1.5)	1 (0.9)	2 (2.4)
History of previous TB treatment, n (%)	Yes	34 (16.9)	16 (13.6)	18 (21.7)
	No	129 (64.2)	74 (62.7)	55 (66.3)
	Unknown	38 (18.9)	28 (23.7)	10 (12.0)

IQR: Interquartile range, HIV: Human immunodeficiency virus, BMI: Body mass index, TB: Tuberculosis

Table 2

Univariate logistic regression for the risk of infection caused by *M. tuberculosis* Lineage 2 strains (n=112).

Explanatory variable	**Lineage 4 n=84 (75.0%)**	**Lineage 2 n=28 (25.0%)**	**Associations with *M. tuberculosis* Lineage 2**	
			OR (95% CI)	**p-value**
Sex, n (%)				
*Women	32 (38.1)	15 (53.6)		
Men	52 (61.9)	13 (46.4)	0.5 (0.2-1.3)	0.15
Age – median years (IQR)	26 (20-31)	28 (21-34)	1.0 (0.9-1.1)	0.79
PNG province of origin, n (%)				
Madang	37 (48.7)	10 (52.6)	0.9 (0.3-2.9)	0.82
East Sepik	23 (30.3)	4 (21.1)	0.6 (0.1-2.4)	0.43
*Other	16 (21.0)	5 (26.3)		
HIV, n (%)				
*Negative	70 (94.6)	23 (95.8)		
Positive	4 (5.4)	1 (4.2)	0.8 (0.1-7.2)	0.81
Negative sputum at enrollment, n (%)				
*No	80 (96.4)	26 (92.9)		
Yes	3 (3.6)	2 (7.1)	2.1 (0.3-13.0)	0.45

*The asterisk indicates the reference category. OR: Odds ratio, CI: Confidence interval, IQR: Interquartile range, PNG: Papua New Guinea, HIV: Human immunodeficiency virus.

Table 3

Spoligotypes for the 3 observed lineages (n=173).

Lineage n (%)	Spoligotype pattern	SIT	Spoligotype family	n (%)
Lineage 4	■■■■■■■■■■■■■□■■■■■■■■■■■■■■■■■□□□□■■■■■■■	393	T1	39 (22.5)
133 (76.9)	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	53	T1	26 (15.0)
	■■■■■■■■■■■■□□□□■■■■■■■■■■■■■■■■■□□□□■■■■■■■	102	T1	16 (9.2)
	■■■■■■■■■■■■■■■■■□■■■■■■■■■■■■■■■□□□□■■■■■■■	119	X1	10 (5.7)
	■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■■■□□□□■■■■■■■	42	LAM9	10 (5.7)
	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■□□□□	51	T1	2 (1.1)
	■□■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	334	T1	1 (0.6)
	■■■■■■□■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	926	T1	1 (0.6)
	■■■■■■■■■□■■■■□■■■■■■■■■■■■■■■■■□□□□■■■■■■■	1475	T1	1 (0.6)
	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■□■■	612	T1	1 (0.6)
	■■■■■■■■■■■■□□■■■■■■■■■■■■■■■■■■□□□□■■■□■■■	875	T2	1 (0.6)
	■■■■■■■■■■■■■■□■■■■■■■■■■■■■■■■■□□□□■■■■■■■	118	T2	1 (0.6)
	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■□■■■	52	T2	1 (0.6)
	■■■■■■■■■■■■□■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	37	T3	1 (0.6)
	■■■■■■■■■■■■■■■■■□■■■□□■■■■■■■■■□□□□■□□■■■■	39	T4-CEU1	1 (0.6)
	■■■■■■■■■■■■■■■■■□■■■■■■■■■■■■■■■□□□□■■■■■■■	40	T4	1 (0.6)
	■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■■■□□□□■■□■■■■	162	LAM9	1 (0.6)
	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■■■■■■■	50	H3	1 (0.6)
	■■■■■■□■■■■■■□■■■■■■■■■■■■■■■■■□□□□■■■■■■■	-	Orphan	3 (1.7)
	■■□■■■■■■■■□■■■■■■■□□□□■■□□■■■■□□□□■■■□■■■	-	Orphan	2 (1.1)
	■■■■□□□□■■■■■■□□■■■■■■■■■■■■■■■□□□□■■■■■■■	-	Orphan	2 (1.1)
	■■■■■■■■■■□□□□□□□□□□□□□□■□□□□□□■□□□□■■■■■■■	-	Orphan	2 (1.1)
	■□■■■■■■■■■■■□■■■■■■■■■■■■■■■■■□□□□■■■■■■■	-	Orphan	1 (0.6)
	■■□□■■■■■■■■■□■■■■■■■■■■■■■■■■■□□□□■■■■■■■	-	Orphan	1 (0.6)
	■■■□■■■■■■■■■■■■■□■■■□□■■■■■■■■■□□□□■□□■■■■	-	Orphan	1 (0.6)
	■■■■□□□□□□□□□□□□■□■■■■■■■■■■■■■■■□□□□■■■■■■■	-	Orphan	1 (0.6)
	■■■■■■□□□□□□□□□□□□□□□□□□□□□□□□□□■□□□□■■■■■■■	-	Orphan	1 (0.6)
	■■■■■■■■■■■□□□□□■■■■■■■■■■■■■■■■■□□□□■■■■■■■	-	Orphan	1 (0.6)
	■■■■■■■■■■■■■□■■■■■■■■■■■□■■■■■□□□□■■■■■■■	-	Orphan	1 (0.6)
	■■■■■■■■■■■■■□■■■■■■■■■■■■■■■■■□□■■■■■■■■■	-	Orphan	1 (0.6)
	■■■■■■■■■■■■■■■■■■■■■□□□□□□□□□□□□□□□□□□■■■	-	Orphan	1 (0.6)
Lineage 2	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■	1	Beijing	38 (22.0)
49 (22.5)	■■■■■■■■■■■■■□□■■■■■■■■■■■■■■■■■■■■■■■■■■■■	-	Orphan	1 (0.6)
Lineage 1	■■□■■■■■■■■■■■■■■■□□■■■■■■■□□□□■□■■■■■■■■■		EAI	1 (0.6)
1 (0.6)				

SIT: Shared international type

Table 4

Drug resistance patterns observed among non pan-susceptible isolates (n=27).

Resistance type n		**Lineage 4 (%) n=12**	**Lineage 2 (%) n=15**	**Total n=27**
Monoresistant	RIF	0	1 (6.7)	1 (3.7)
15	INH	6 (50.0)	3 (20.0)	9 (33.3)
	STR	2 (16.7)	2 (13.3)	4 (14.8)
	PYZ	1 (8.3)	0	1 (3.7)
Polyresistant	INH+STR	0	3 (13.3)	3 (11.1)
3				
MDR	RIF+INH	0	5 (30.0)	5 (18.6)
9	RIF+INH+ETD	2 (16.7)	0	2 (7.4)
	RIF+INH+STR+ETD	0	1 (6.7)	1 (3.7)
	RIF+INH+STR+ETD+PAS	1 (8.3)	0	1 (3.7)

MDR: multidrug-resistant, RIF: rifampin, INH: isoniazid, STR: streptomycin, PYZ: pyrazinamide, ETD: ethionamide, PAS: p-amino salicylic acid.

Table 5

Univariate logistic regression for the risk of resistance to at least one anti-TB drug (n=112).

Explanatory variable	**Pan-susceptible n=94 (83.9%)**	**Resistant n=18 (16.1%)**	**Associations with resistance to ≥1 drug**	
			OR (95% CI)	**p-value**
Sex, n (%)				
*Women	38 (40.4)	9 (50.0)		
Men	56(59.6)	9 (50.0)	0.7 (0.2-1.9)	0.45
Age – median years (IQR)	25 (20-30)	29 (24-35)	1.0 (1.0-1.1)	0.05
PNG province of origin, n (%)				
Madang	41 (49.4)	5 (41.7)	0.4 (0.1-1.5)	0.18
East Sepik	26 (31.3)	2 (16.6)	0.2 (0.04-1.4)	0.12
*Other	16 (19.3)	5 (41.7)		
History of previous TB treatment, n (%)				
*No	61 (83.6)	9 (75.0)		
Yes	12 (16.4)	3 (25.0)	1.7 (0.4-7.2)	0.48
HIV, n (%)				
*Negative	78 (95.1)	15 (93.8)		
Positive	4 (4.9)	1 (6.2)	1.3 (0.1-12.2)	0.82
Strain lineage, n (%)				
*Lineage 4	75 (80.7)	8 (44.4)		
Lineage 2	18 (19.3)	10 (55.6)	5.2 (1.8-15.1)	< 0.01
Negative sputum at enrollment, n (%)				
*No	88 (94.6)	18 (100)		
Yes	5 (5.4)	0 (0)	Not defined	

*The asterisk indicates the reference category. OR: Odds ratio, CI: Confidence interval, IQR: Interquartile range, PNG: Papua New Guinea, TB: Tuberculosis, HIV: Human immunodeficiency virus.

Table 6

Univariate logistic regression for the risk factors associated with molecularly clustered *M. tuberculosis* strains (n=162).

Explanatory variable	**Non-clustered n=90 (55.6%)**	**Clustered n=72 (44.4%)**	**Associations with molecularly clustered strains**	
			OR (95% CI)	**p-value**
Sex, n (%)				
*Women	18 (34.6)	26 (52.0)		
Men	34 (65.4)	24 (48.0)	0.5 (0.2-1.1)	0.08
Age – median years (IQR)	26 (20-30)	26 (21-34)	1.0 (1.0-1.1)	0.42
PNG province of origin, n (%)				
Madang	21 (43.8)	22 (57.8)	1.4 (0.5-4.3)	0.51
East Sepik	16 (33.3)	8 (21.1)	0.7 (0.2-2.4)	0.56
*Other	11 (22.9)	8 (21.1)		
HIV, n (%)				
*Negative	44 (95.7)	39 (92.9)		
Positive	2 (4.3)	3 (7.1)	1.7 (0.3-10.7)	0.58
Reported previous TB medication, n (%)				
*No	34 (79.1)	28 (84.9)		
Yes	9 (20.9)	5 (15.1)	0.7 (0.2-2.2)	0.52
Strain lineage, n (%)				
*Lineage 4	42 (82.4)	35 (70.0)		
Lineage 2	9 (17.6)	15 (30.0)	2.0 (0.8-5.1)	0.15

*The asterisk indicates the reference category. OR: Odds ratio, CI: Confidence interval, IQR: Interquartile range, PNG: Papua New Guinea, HIV: Human immunodeficiency virus, TB: Tuberculosis.

Chapter 2

Drug resistance-conferring mutations in *Mycobacterium tuberculosis* isolates from Madang, Papua New Guinea

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Summary

Monitoring drug resistance in *Mycobacterium tuberculosis* is essential to curb the spread of tuberculosis (TB). Unfortunately, drug susceptibility testing is currently not available in Papua New Guinea (PNG) and that impairs TB control in this country. We report for the first time *M. tuberculosis* mutations associated with resistance to first and second-line anti-TB drugs in Madang, PNG. A molecular cluster analysis indicated evidence for transmission of resistant *M. tuberculosis* in that region.

Introduction

Mycobacterium tuberculosis drug resistance is a global concern. In Papua New Guinea (PNG), the estimated tuberculosis (TB) incidence rate was 303/100000 population in 2010, with estimations for 5% and 24% multidrug resistant TB (MDR-TB) among new and retreatment cases, respectively (1). Culture-based drug susceptibility testing (DST) requires laboratory infrastructures, which are often too sophisticated for resource-constrained settings like PNG. Detecting resistance-associated mutations is a faster alternative, as shown by GenoType MTBDR*plus* (Hain Lifescience) (2) or the recently endorsed Xpert MTB/RIF (Cepheid) (3).

In order to monitor drug resistance molecularly, the distribution and frequency of the relevant drug resistance-conferring mutations in a given setting needs to be known, however no such data is currently available for PNG. In this study, we report mutations associated with drug resistance among pulmonary TB isolates collected in the Madang area of PNG. In addition, we provide evidence for primary transmission of drug-resistant *M. tuberculosis* strains.

Patients and Methods

The study settings, patient characteristics and *M. tuberculosis* genotypes were reported elsewhere (M. Ballif *et al*., manuscript submitted). In brief, patients at least 15 years old visiting the TB clinic of Madang were included if having microscopically confirmed pulmonary TB or other clinical evidence for smear-negative TB.

New TB patients were treated with isonazid (INH), rifampicin (RIF), ethambutol (EMB) and pyrazinamide (PZA) for two months, followed by four months with INH and RIF. Previously treated patients were given INH, RIF, EMB, PZA and streptomycin (STR) for two months, followed by one month with INH, RIF, EMB, PZA and five months with INH, RIF and EMB.

DST was performed by proportion method at the Queensland Mycobacterial Reference Laboratory in Australia using BACTEC MGIT 960 (Beckton Dickinson), as previously described (Ballif *et al.*, manuscript submitted).

Among resistant isolates, the following genes (or gene regions) were sequenced: *katG*, *inhA* promoter, *ahpC* promoter, *rpoB*, *embB*, *pncA*, *rpsL*, *rrs*, *gidB*, and *gyrA*. Primers and PCR conditions are given in Supplementary table 1.

Clusters were defined for strains sharing identical spoligotype and 24 locus locus mycobacterial interspersed repetitive unit variable number of tandem repeats (MIRU-VNTR) patterns. Strain lineages were obtained by real-time PCR identification of lineage-specific SNPs, as previously described (4–7).

The National Medical Research Council's Ethics Committee of PNG and the Ethikkommission Beider Basel in Switzerland approved the study protocol. Written informed consent was obtained from all patients enrolled in the study.

Results and Discussion

Sixty patients were recruited in the frame of a pilot study in 2005-2007 and 201 in the frame of a treatment cohort study in 2009-2010. History of previous TB treatment was reported in 16.9% (31/201) of the 2009-2010 patients, for whom data was collected.

Molecular analyses were performed on DNA from 173 successfully grown isolates and phenotypic DST was obtained for 172 of them.

Overall, 27/172 isolates were resistant to $\geq$ 1 drug: 15/172 (8.7%) were monoresistant, 3/172 (1.8%) polyresistant and 9/172 (5.2%) MDR. A total of 10/172 (5.8%) were RIF resistant isolates, 21/172 (12.2%) INH resistant (13 low-level [0.1 mg/L], 8 high-level [0.4 mg/L]), 9/172 (5.2%) STR resistant, and 4/172 (2.3%) ethionamide (ETD) resistant. The observed resistance-conferring mutations are listed in table 1.

INH resistant isolates harbored mutations in *katG* (codon S315T) or *inhA* promoter (nucleotide C15T). All but one isolates with *katG* S315T were resistant to 0.4 mg/L INH, whereas all isolates with *inhA* promoter mutation were sensitive at this drug concentration (but resistant to 0.1 mg/L INH), thus confirming the association between *inhA* promoter mutations and low-level INH resistance (8). Among all the 6/9 MDR-TB isolates with either *katG* or *inhA* promoter mutations, all had the *katG* S315T mutation, except one with an *inhA* promoter mutation. This only MDR-TB case harboring an *inhA* promoter mutation was one of the four MDR-TB cases additionally resistant to ETD. Mutations in *inhA* promoter have been shown to cause INH and ETD cross-resistance and were thereby associated with higher risks of XDR-TB (9).

Eight INH resistant strains (38.1%) had no *katG* or *inhA* promoter mutation. Only 850bp of *katG* were sequenced and therefore some mutations may have been missed. However, *katG* mutations outside this region are comparably rarer (8,10,11). Alternatively, these strains might harbor mutation(s) in the > 20 other genes reported as potentially associated with INH resistance (10).

We did not observe any *ahpC* promoter mutation, thought to compensate the reduced catalase-peroxidase activity resulting from *katG* mutations (12,13). Because the INH resistance-conferring mutations observed here, i.e. *katG* S315T and *inhA* promoter C15T, are known to be associated with a low fitness cost (13), they might not require compensation.

All RIF resistant isolates harbored mutations in *rpoB* at codons D516F, D516Y or S531L, except one which did not have any mutation in the 600bp *rpoB* fragment sequenced. DST was repeated for this case, confirming the MDR phenotype. Furthermore, common *rpoB*, *katG* and *inhA* promoter mutations were excluded by GenoType MTBDR*plus* assay (Hain Lifescience). Mutation S531L has frequently been linked to high-level RIF resistance (14), whereas D516Y was recently associated with low-level resistance (15–17). Mutation D516F has only been reported in Kazakhstan (18) and may also cause low-level resistance. Low-level RIF resistance has been little considered so far, but could influence treatment, especially knowing that phenotypic DST outcomes may differ from the actual efficacy of the anti-TB drugs in patients (19).

STR resistant isolates harbored mutations in *rpsL* (codons K43R, K88Q, K88R) and *rrs* (nucleotide A514C), as previously reported (20,21). In addition, one isolate was mutated at codon V77G in *gidB*, a mutation which was not reported before. One STR resistant isolate did not present any mutation in these genes.

Mutations in *gidB* have been associated with low-level STR resistance (22,23), but were also reported in sensitive strains (24). In this study, *gidB* mutations A10P, L16R, E92D, and A205A were observed among strains resistant to other drugs than STR. We further explored *gidB* mutations in whole genomes of 21 pan-susceptible strains representative of the six defined *M. tuberculosis* lineages (25). The mutation *gidB* V77G, which we observed in one STR resistant isolate from PNG, could not be found in any of the 21 pan-susceptible strains analyzed. This mutation could therefore indeed be involved in drug resistance or could be a transitory polymorphism in the population. The mutation A10P observed in one STR sensitive isolate was not found in any of the 21 pan-susceptible genomes. The mutations L16R was observed in genome sequences from Lineage 4 strains (Euro-American lineage) and E92D in Lineage 2 strains (East-Asian lineage, includes Beijing genotype). This supports the recent observation that *gidB* L16R occurs in LAM strains (i.e. Lineage 4), whereas *gidB* E92D occurs in Beijing strains (26). A205A appeared as mutated in all strains not belonging to Lineage 4, therefore indicating that this mutation, identified by comparison to H37Rv, is in fact a mutation of Lineage 4. Observations from the 21 pan-susceptible genomes thereby suggest that most *gidB* mutations rather reflect *M. tuberculosis* lineage evolution than drug resistance.

No mutations were observed in genes *embB*, *pncA*, or *gyrA*, which are associated with EMB, PZA or fluoroquinolone resistance, respectively.

Among isolates with complete spoligotyping and MIRU-VNTR data, 72/162 (44.4%) were clustered. Despite potential fitness costs associated with resistance-conferring mutations (27), the proportion of clustered strains was not significantly different among drug-sensitive (60/137, 43.8%) and drug-resistant (12/25, 48.0%) isolates.

To distinguish between primary resistance and acquired resistance, clustered isolates sharing identical drug resistance-conferring mutations were considered. Five of the 12 (41.7%) drug-resistant isolates involved in molecular clusters shared their drug resistance-conferring mutations with other isolates in the same cluster, thus strongly suggesting patient-to-patient transmission.

This study provides so far missing data about drug resistance-conferring mutations in *M. tuberculosis* isolates from Madang in PNG. Monitoring drug resistance is essential to prevent the spread of resistant bacteria, especially in diseases requiring lengthy treatments such as TB. Our data suggests that not all the existing drug resistance associated mutations may be detected by molecular tests, which mainly focus on a subset of polymorphisms only. However, given the complex implementation of culture-based DST, PNG may be well suited for an accelerated roll-out of molecular drug resistance testing in order to better tackle the emergence and the transmission of drug-resistant *M. tuberculosis* strains.

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Tables

Table 1

List of all mutations observed in each of the 27 strains resistant to at least one drug.

		rpoB D516F	*rpoB* D516Y	*rpoB* S531L	*katG* S315T	*inhA* (promoter) C15T	*rpsL* K43R	*rpsL* K88Q	*rpsL* K88R	*rrs* A514C	*gidB* V77G	*gidB* A10P	*gidB* L16R	*gidB* E92D	*gidB* A205A
1	RIF monoresistant			■										■	
2	INH monoresistant				■									■	
3	INH monoresistant				■									■	
4	INH monoresistant					■						■			■
5	INH monoresistant					■						■			■
6	INH monoresistant												■		■
7	INH monoresistant													■	
8	INH monoresistant														■
9	INH monoresistant														■
10	INH monoresistant														■
11	STR monoresistant								■					■	
12	STR monoresistant									■				■	
13	STR monoresistant										■				■
14	STR monoresistant														■
15	PZA monoresistant														■
16	INH + STR polyresistant					■	■							■	
17	INH + STR polyresistant					■	■							■	
18	INH + STR polyresistant					■	■							■	
19	RIF + INH MDR	■			■									■	
20	RIF + INH MDR	■			■									■	
21	RIF + INH MDR	■			■									■	
22	RIF + INH MDR		■		■									■	
23	RIF + INH MDR			■										■	
24	RIF + INH + ETH MDR			■		■						■			■
25	RIF + INH + ETH MDR			■											■
26	RIF + INH + ETH + STR MDR									■				■	
27	RIF + INH + ETH + STR + PAS MDR		■		■			■							■

The polymorphisms are indicated at codon positions, except for *rrs* gene. RIF: Rifampin; INH: Isoniazid; STR: Streptomycin; PZA: Pyrazinamide; ETH: Ethionamide; PAS: *p*-aminosalicylic acid; MDR: Multidrug resistant

Supplementary table 1

Primers and PCR conditions.

Gene		Direction	Primer sequence (5'-3')	PCR conditions	Product length (bp)	Reference
rpoB	Rv0667	Forward	TCGGCGAGCTGATCCAAAACCA	Tm: 62°C, Ex: 45, Cy: 35	601	This study
rpoB	Rv0667	Reverse	ACGTCCATGTAGTCCACCTCAG			This study
inhA (promoter)	Rv1484	Forward	GGCACGTACACGTCTTTATGTA	Tm: 60°C, Ex: 30, Cy: 35	478	[1]
inhA (promoter)	Rv1484	Reverse	GGTGCTCTTCTACCGCCGTGAA			[1]
katG	Rv1908c	Forward	CCAGCGGCCCAAGGTATC	Tm: 64°C, Ex: 60, Cy: 35	850	This study
katG	Rv1908c	Reverse	GCTGTGGCCGGTCAAGAAGAAGTA			This study
ahpC (promoter)	Rv2428	Forward	ACCACTGCTTTGCCGCCACC	Tm: 64°C, Ex: 30, Cy: 35	236	[2]
ahpC (promoter)	Rv2428	Reverse	CCGATGAGAGCGGTGAGCTG			[2]
embB	Rv3795	Forward	CGGCATGCGCCGGCTGATTC	Tm: 65°C, Ex: 30, Cy: 35	260	[3]
embB	Rv3795	Reverse	TCCACAGACTGGCGTCGCTG			[3]
gyrA	Rv0006	Forward	CAGCTACATCGACTATGCG	Tm: 58°C, Ex: 30, Cy: 35	320	[4]
gyrA	Rv0006	Reverse	GGCTTCGGTGTACCTCATC			Adapted from [4]
gidB	Rv3919c	Forward	CGAGAGCGGAGAATGTTTCAC	Tm: 60°C, Ex: 60, Cy: 35	793	This study
gidB	Rv3919c	Reverse	CTGGCCCGACCTTACGAGC			This study
rpsL	Rv0682	Forward	CGTGAAAGCGCCCAAGATAG	Tm: 62°C, Ex: 30, Cy: 35	333	This study
rpsL	Rv0682	Reverse	GAACCGCGGATGATCTTGTAG			Adapted from [5]
rrs	MTB000033	Forward	GATGACGGCCTTCGGGTTGT	Tm: 60°C, Ex: 30, Cy: 35	238	[5]
rrs	MTB000033	Reverse	TCTAGTCTGCCCGTATCGCC			[5]
rrs	MTB000033	Forward	GTAGTCCACGCCGTAAACGG	Tm: 60°C, Ex: 30, Cy: 35	245	[5]
rrs	MTB000033	Reverse	CACACAGGCCACAAGGGAAC			Adapted from [5]
rrs	MTB000033	Forward	CGTTCCCTTGTGGCCTGTG	Tm: 62°C, Ex: 45, Cy: 35	547	This study
rrs	MTB000033	Reverse	GGCGTTTTCGTGGTGCTCC			Adapted from [5]
pncA	Rv2043c	Forward	GGCTGCCGCGTCGGTAGG	Tm: 62°C, Ex: 45, Cy: 35	652	This study
pncA	Rv2043c	Reverse	GCCGCCAACAGTTCATCCC			This study

All PCRs were performed using FastStart Taq DNA Polymerase and the corresponding buffers (Roche Diagnostics, Switzerland). Tm: Annealing temperature, Ex: Extension time (seconds), Cy: number of cycles.

References to Supplementary table 1:

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General discussion

Synopsis of findings

The aim of this thesis was to provide so far missing but necessary information about the characteristics of *M. tuberculosis* strains circulating in PNG, with focus on the Madang region. The analyses of patient sputum isolates collected in this area indicated substantial rates of drug-resistant TB. Overall, 15.7% of the strains were resistant to at least one drug and 5.2% were MDR. Furthermore, the genotyping of these strains indicated the presence of two predominant *M. tuberculosis* lineages: about three quarters Euro-American lineage (i.e. Lineage 4) and about one quarter East-Asian lineage (i.e. Lineage 2). Samples of the East-Asian lineage were more often found to be associated with drug resistance, as previously observed. The lineages were sub-divided into distinct spoligotypes. While Lineage 2 was composed of two different spoligotypes, Lineage 4 comprised 31 different spoligotypes, thus illustrating the actual diversity that *M. tuberculosis* can harbor. This diversity was used to define molecular clusters of *M. tuberculosis* strains by combining the DNA fingerprints obtained from spoligotyping and from 24-locus MIRU-VNTR typing. We observed that 44.4% of the strains shared identical DNA genotypes, suggesting evidence for recent transmission. Furthermore, 25.0% of the clusters included drug-resistant strains, indicating that these are circulating in the community as well.

Study design

This study was launched in 2007-2008 when the PNG national TB program asked the PNG IMR to initiate research on TB with the main goal of describing the extent of disease burden and drug resistance. Bringing their respective expertise together, the PNG IMR and the Swiss TPH embarked on this TB project, combining research and capacity transfer objectives in a sustainable way.

Capacity transfer

The transfer of capacity was a primordial component of this thesis, especially at its beginning. Indeed, the results presented here would not have been obtained without the adequate work performed at the TB laboratory in Madang. Time was therefore invested in training local staff, so

that this laboratory could sustainably support TB activities in the region. The primary objective was to implement routine mycobacteriological techniques for the manipulation of patient sputum samples collected at Modilon General Hospital and other collaborating health centers in the region. For this purpose, two visits were scheduled to Madang, during which the Swiss TPH and the PNG IMR collaborators have set up the necessary infrastructures and protocols. Although this capacity transfer component required time investment far beyond the two visit periods and arguably reduced the time allocated to analyses and possibly the amount of results obtained for the present thesis, it was an absolute prerequisite to this project and was considered on its own as part of the thesis.

Study site

The newly available laboratory offered a unique opportunity to initiate the present project in Madang. Indeed, apart from one laboratory in Port Moresby, the capital city of PNG, no other laboratory in PNG was equipped for sputum handling and therefore, the building of these new facilities was decisive for the initiation of the study in Madang. Furthermore, this project was made possible by the availability of a local medical team willing to get involved and support this project on a long term basis. However, because strain culture cannot be done in a biosafety level 2 laboratory, the QMRL (in Australia) was involved in the study for the culture and phenotypic DST on all collected isolates.

The size and importance of the Modilon General Hospital for Madang and surroundings could ensure that active TB patients would primarily seek care in this hospital. Nevertheless, one cannot exclude that this created a bias towards possibly sicker individuals and thereby higher rates of MDR-TB. To confirm this possible bias, data from longitudinal drug resistance monitoring and multi-site comparisons of drug resistance rates in PNG would be needed. Furthermore, it is also possible that the proximity of the newly available laboratory and the initiation of the present study influenced the suspicion for TB among both the patients and the medical staff in that region. However, if the latter is true, we can suppose that this did not necessarily bias the study population towards only sicker individuals.

Knowing that the Modilon General Hospital is the most important hospital in the region of Madang, a fairly large number of TB patients was expected (about 300 patients per year). In the end, the number of patients included in the study period was lower, with about 200 pulmonary TB patients recruited between April 2009 and October 2010. Although we do not have an explanation for this, we speculate that various factors could influence the number of pulmonary patients

observed in the frame of this study. It could well be that pulmonary TB is not that frequent in this region. Nevertheless, the existence of molecularly clustered *M. tuberculosis* strains suggests that tubercle bacilli might be circulating in the population, and that at least part of the observed active TB cases likely resulted from recent transmission. Another factor could be related to the study design (also discussed below), which in this situation was based on the passive detection of suspected TB patients seeking care. This approach relies largely on the awareness of TB in the population, which may not be equal everywhere. Although more specific investigations would be necessary to address this point, one could speculate that TB awareness may for example depend on the patients' living place, level of education, socio-economic status, religion and other beliefs. Furthermore, the symptoms of active TB are not particularly specific and may be mistaken for less severe conditions, such as cold or flu, which may not lead the patient to seek adequate health care. Lastly, refusal to participate in the study or withdrawals may also have impacted on the final patient number.

Inclusion criteria

The inclusion criteria required patients aged above 14 years with microscopically confirmed pulmonary TB or other clinical evidence suggesting smear-negative TB. The decision of not including younger patients was supported by the fact that children represent a distinct category of patients with less sputum production (making diagnosis difficult) and more frequent extra-pulmonary TB [105]. For these two reasons, childhood TB presumably contributes little to TB transmission and is often not primarily investigated. However, including younger pulmonary TB patients in this study could have re-enforced the cohort size, possibly revealing interesting aspects related to the disease outcome or the patient-to-patient transmission, when compared to the adult patients.

Smear-negative TB (i.e. culture-confirmed TB patients for whom sputum smear microscopy could not detect any tubercle bacillus) is an important challenge in TB monitoring. The sensitivity of smear microscopy is low (about 50-60% or even less in low-income countries [106]) and therefore, samples containing bacilli below the level of detection may be classified as TB negative. However, if TB is strongly suspected based on other clinical evidence (for example chest X-ray or chronic cough), the patient can be classified as smear-negative TB and ideally, the presence of mycobacteria should be confirmed by culture. Smear-negative TB is often neglected due to the intrinsic lower risk of transmission, reduced disease extent and good prognosis [106]. In the absence of confirmation by *in vitro* culture, the adequate selection of criteria to define smear-

negative TB is essential. As an illustration, a study conducted in Zimbabwe indicated that cough for more than three weeks (chronic cough) most likely indicates TB and is therefore a good criterion to select for the diagnosis of smear-negative TB in the absence of culture facilities [107]. Interestingly, this study also showed that in this setting, the culture of smear-negative specimens added only little information to the microscopy-based diagnosis.

In our study, only a few smear-negative TB patients were included (13 smear-negative patients between April 2009 and October 2010, two of them were HIV positive and culture was successful for five of them). Therefore, no particular analysis could be performed on this group of patients in the frame of this thesis. If numbers allowed, it would have been interesting to address questions related to smear-negative TB, for example to validate the clinical suspicion for TB with *in vitro* culture, to identify the best clinical predictors for TB in case of negative sputum, to see if any patient demographic or clinical characteristic is a risk for smear-negative TB or to investigate the treatment outcomes among these patients.

Study limitations

The major limitation of this thesis was the low number of patients included, or more precisely, the low number of samples available for molecular and statistical analyses. Although the TB clinic of the Modilon General Hospital was important enough to attract numerous patients in the region, each manipulation step from the patient sputum collection to the DNA extraction might have been a potential source of error, thereby impacting on the final number of samples available. First, the sputum needed to be stored adequately until decontamination, in order to avoid excessive contamination and to maintain the bacilli viable for culture. The decontamination process aims at killing bacteria other than mycobacteria and present in the sputum, while liquefying the sputum to make most tubercle bacilli accessible. For that, the right balance of reagents is needed and a contamination rate of 3–5% is considered as a good balance between killing contaminants and keeping the mycobacteria alive [30]. The quality of the reagents and the accuracy of each steps in the protocol are crucial for successful bacterial cultures and ideally, decontaminated sputum should directly be inoculated into the culture medium and incubated. This was unfortunately not always the situation faced by the TB laboratory in Madang. Decontamination might sometimes have only been performed when sufficient numbers of sputum samples were collected and inoculated growth tubes might have been stored until a sufficient number of samples could be shipped to the QMRL. Culture contamination and absence of growth were frequent, and all the above-listed steps could possibly be incriminated. Although some contaminated cultures could be rescued by a second

decontamination process to recover living mycobacteria, this was not always successful. In contrast, the absence of growth indicated that viable mycobateria were either killed during decontamination or were in fact absent from the sputum (TB negative patient). In such situations, a second sample would have been necessary for validation, although backup samples were mostly not available. Finally, the method selected for DNA extraction might impact on the final DNA amount and quality. The protocol adopted in this project (i.e. a commercial chelating resin) is fast and easy to perform on grown cultures, but possibly not yielding DNA of high quality. Although this should have been sufficient for PCRs, we observed that some extracts were not amplifiable and therefore not usable for molecular investigations. Other protocols might have improved the DNA quality but would have certainly been more time consuming (and costly) than the selected one.

Another limitation of the present study was the absence of contact tracing data, which could have provided useful information for the interpretation of *M. tuberculosis* transmission. Combining precise contact tracing information with molecular cluster data would have allowed a more accurate evaluation of recent transmission, ensuring that patients infected by *M. tuberculosis* strains sharing an identical genotype have truly been in contact. Furthermore, elucidating where and how transmission of TB occurred among clusters could have potentially provided useful information to better tackle transmission at these precise occasions. However, contact investigations can be complex, since they imply the identification and assessment of possible contacts of each sputum smear positive TB cases, starting within households and expanding to contacts outside households. Furthermore, ethical considerations play an important role when secondary TB cases are identified [108].

Finally, it is possible that an approach based on active case finding would have ensured a larger study population by recruiting active TB patients who may not have come to the clinic otherwise. In fact, this is the approach implemented in the continuation phase of this project, whereby population screenings are organized to detect TB patients in various locations in PNG. The feasibility of active case finding in PNG depends largely on the availability of the necessary staff and infrastructure to conduct such studies, including microscopists and microscopes, sputum storage and transport to Madang for decontamination. Compared to passive case finding, which mimics the usual patient care structure, active case finding requires intensive availability of skilled staff for short periods and at precise locations. This may therefore only be suitable for short term investigations and not for usual care.

Implications for tuberculosis control

Drug resistance

The observed rates of drug-resistant TB were substantial in the Madang area, indicating that a treatment based on regular first-line drugs cannot guarantee patient cure in all cases. MDR-TB patients require second-line therapies which are long and expensive. Furthermore, patient isolation would be recommendable in case of MDR-TB to limit potential transmission to healthy individuals, but this is seldom feasible in settings like in PNG.

Our results indicated that not only RIF and INH resistance were circulating in the Madang area but also STR and ETD. This is useful information for the management of patient treatment since STR is commonly added to first-line regimens when evidence for relapse exists in the absence of DST outcomes. Resistance to ETD (one of the second-line drug option) is often emerging together with INH resistance because both drugs target the same carrier protein reductase (InhA) [109]. The risk of cross-resistance indicates that this drug may often not be a good option for second-line therapies, especially in the presence of known *inhA* promoter mutations.

Various aspects of patient management can contribute to the development of drug resistance. Typically, the acquisition of resistance can occur during a treatment course if the drug dosage is sub-optimal, if the drug quality is not as it should be, if compliance to medication is poor or in case of interaction with other drugs reducing treatment efficacy. If these aspects are left unnoticed and if resistance develops, the patient remains infective and transmission of already resistant *M. tuberculosis* to other susceptible individuals can occur (primary resistance) in an uncontrolled way.

MDR-TB often remains undetected for prolonged periods when DST and other TB diagnostic tools are not readily available on site. And even if available, DST is a lengthy process due to the slow growth rate of *M. tuberculosis* and patients may be treated with a sub-optimal regimen in the meantime. As an alternative, rapid point-of-care molecular tools applicable on sputum directly would be advantageous in these situations. Ideally, such tools should be easy to perform, fast and cheap. Xpert MTB/RIF (Cepheid, USA) offers a quick and simple way of combining TB diagnosis and RIF resistance test, circumventing the needs for culture or other particular laboratory equipment and know-how [41]. The routine use of such tools in PNG could be of immense benefit, allowing drug resistance to be detected already on the first day of clinical visit and therefore to embark directly on adequate therapy if necessary. At the moment, the cost of this technology is still prohibitive for resource-constrained settings like PNG, especially compared to microscopy.

However, substantial price reductions are being negotiated to make Xpert MTB/RIF affordable in low- and middle-income countries [41]. In early 2011, the estimated price per test was still substantially greater than for microscopy, but similar to what culturing and DST cost [41]. Faster tests, based for example on a dipstick principle would be ideal, but unfortunately do not exist yet.

Transmission

An elevated proportion of molecular clusters was observed in the region of Madang, comprising both drug-susceptible and drug-resistant *M. tuberculosis* strains. On the one hand, transmission of *M. tuberculosis* can occur before active TB is diagnosed, that is when an infectious patient does not know yet about his or her condition and transmits TB to his or her contacts. On the other hand, transmission can occur during the course of treatment, if the prescribed regimen or the adherence is not appropriate and the infection is not cleared efficiently. Active case finding could be a way of reducing transmission by increasing prompt TB diagnosis in the asymptomatic population. Although screening the whole population is not possible, populations at higher risk for TB may need to be targeted first, for example people living or working in particularly crowded places, where transmission is facilitated, or HIV-infected people.

When recent transmission can reasonably be ruled out by molecular cluster analysis or contact tracing, the presence of active TB in principle indicates the reactivation of latent TB. It is important to keep in mind that about one third of the world population is latently infected with *M. tuberculosis* and in average, about 10% of these individuals will develop active TB in their lifetime, thereby becoming potential transmitters [21]. However, this proportion is higher in some risk groups. Typically, HIV is the strongest known risk factor for TB and co-infected individuals are 21 to 34 times more likely to develop active TB [96]. The impact of HIV in PNG is certainly not comparable to situations such as observed in Sub-Saharan Africa, although PNG is estimated to be the country with the highest HIV prevalence in Oceania: 0.9% [110]. Other conditions such as smoking, excessive alcohol consumption, malnutrition, diabetes mellitus or indoor air pollution have also been linked to higher risks of active TB and may be more relevant for PNG [111–113]. These risk factors often remain underestimated in the clinical management of patients, but they may silently contribute to higher rates of latent TB activation and thus to potential transmission. Data on the current extent of non-communicable diseases in PNG is scarce and largely relies on estimates with a high degree of uncertainty. However, according to the WHO latest report, 13.3% of the Papua New Guineans had a raised blood glucose rate in 2008 and diabetes was estimated to account for 2% of all deaths [114]. In fact, the epidemic of type 2 diabetes is thought to be increasing in PNG, probably as a result of

growing westernization and urbanization, and may become a major public health concern in the future [115]. In addition, 40.9% of the Papua New Guineans declare smoking tobacco daily [114], which could also contribute to higher rates of active TB.

Genetic diversity

The genetic diversity observed among the different *M. tuberculosis* lineages has long been underestimated and yet, such diversity might have implications for the future of TB control. In particular, the strain-to-strain genetic variations may be reflected in the efficacy of newly developed (and urgently needed) diagnostic tools, vaccines or anti-TB drugs. An illustration can already be given by the observed geographical differences in BCG efficacy, which is known to be lower among adults in tropical countries [11]. Although several factors may be involved, this poor efficacy might in fact be associated with the phylogeographical differences in *M. tuberculosis* population structure, whereby different *M. tuberculosis* lineages predominate in particular geographical regions [73]. Although this hypothesis needs confirmation, the development of new vaccines should take into account that genetic differences among *M. tuberculosis* lineages exist, and that implications on the global efficacy of vaccine candidates may occur [68].

The genetic diversity of *M. tuberculosis* could also impair diagnostic tools relying on particular DNA sequences, which may not be conserved throughout all lineages. Furthermore, diagnostic tests based on antigen-specific immune response may not perform equally well for all *M. tuberculosis* lineages, as suggested by a study in The Gambia, showing that the T-cell response to particular MTBC antigens varied among lineages [68,116]. Such lineage-dependant differences in immune response may not only have implications for the development of serologic diagnostic tests, but also for vaccine candidates, which should prevent *M. tuberculosis* infections regardless of intrinsic genetic variations.

Regarding anti-TB drugs, a famous difference resulting from the MTBC genetic variation is given by the intrinsic resistance of *M. bovis* to PZA, which is caused by a constitutive mutation in the gene *pncA* [117]. Similarly, other existing lineage-specific mutations may also play a role in intrinsic drug resistances and this may need to be considered for the development of new anti-TB compounds [68]. In addition, *M. tuberculosis* strains belonging to the East-Asian lineage have repeatedly been associated with drug-resistance [118], as reflected by our observations in PNG. Furthermore, the genetic background of *M. tuberculosis* was also shown to influence the propensity of drug-resistant strains to spread effectively [119].

Outlook

This thesis provided the first contribution about the molecular epidemiology of TB in PNG and initiated future research projects on this topic. Since 2010, five additional sites in highland and coastal locations were selected to expand TB research in PNG with an approach combining active and passive TB case finding.

In this section, perspectives about possible research questions left unanswered by this thesis and about the future of TB control in PNG are discussed.

Open questions related to strain genotypes

The genotyping of the strains collected in the frame of this project indicated the presence of mainly two of the six global lineages of human-adapted *M. tuberculosis* described previously [7,73]. One interesting aspect of these lineages is their close association with geographical regions and their preferential occurrence among hosts originating from each of these regions [73]. The two predominant *M. tuberculosis* lineages observed among Papua New Guineans in the Madang area, Lineage 2 and 4, are also called East-Asian and Euro-American for the regions where they prevail. The predominance of Euro-American and East-Asian *M. tuberculosis* lineages in the Madang region could indicate that the introduction of TB in this area is fairly recent and might coincide with the various waves of successive colonization during the last centuries. Along these lines, Lineage 4 *M. tuberculosis* strains (i.e. Euro-American lineage) could have been brought to PNG by Spanish or Portuguese migrants in the 16th century; or possibly later when English settled in PNG at the end of the 19th century, followed by Australians and Germans at the beginning of the 20th century. In addition, many groups of missionaries of European origin have settled in various locations throughout PNG in the last decades and may also have contributed to the spread of Lineage 4 *M. tuberculosis* strains. On the other hand, Lineage 2 *M. tuberculosis* strains (i.e. East-Asian lineage) predominate in Asia and one could hypothesize that they were brought to PNG in parallel to the important immigration of Chinese laborers and traders in the last decades.

The waves of migrations described above may not have reached all regions of PNG to the same extent, and this may be reflected by regional differences in *M. tuberculosis* genotype distribution. For instance, it is well possible that Chinese populations are particularly concentrated in urban regions or around mines and less present in some rural regions of PNG. Such variations might result in higher proportions of the East-Asian lineage of *M. tuberculosis* in these locations. Similarly, we also know that missionaries (generally from European origin) tended to be more numerous in the

coastal regions, which were initially more easily reached than the highlands. It is nevertheless possible that because road and air travel across PNG has become easier in the past decades, the actual regional differences which could have been observed are now faded. Only when data from various locations throughout PNG will be available, will we be able to better assess these hypotheses.

Yet, if different *M. tuberculosis* genotype distributions are seen in particular regions of PNG, one could alternatively hypothesize that these differences mimic the high genetic variation found among indigenous PNG populations. Indeed, PNG has fascinated many researchers in the past decades, especially anthropologists and linguists who were attracted by the great socio-cultural diversity condensed in this country, where some populations evolved over centuries with very limited contacts to the outside world. Linguists have identified more than 800 different Papuan and Austronesian languages in PNG, associated with two separated waves of immigration expanding in PNG about 6,000-10,000 years ago and about 3,300 years ago, respectively [104,120]. Furthermore, molecular analyses showed a great genetic diversity among PNG indigenous populations, particularly marked by a reduced variation within population but a high divergence between populations. The most divergent populations were seen in the rugged interiors of large islands, which are believed to host population groups of older origin [104,120]. If the indigenous populations living in the mountainous regions of PNG (i.e. PNG Highlanders) are genetically distinct from the populations living on the coast, it could be worth exploring whether this difference would also be reflected in the genotypic profiles of the infecting *M. tuberculosis* populations.

In addition, interesting comparisons could be done if *M. tuberculosis* genotypic data from neighboring regions were available. This could for instance include data about strains collected in Australia, other Melanesian countries or Papua, the Indonesian province sharing border with the PNG mainland. Also interesting would be to explore the *M. tuberculosis* genotypes circulating among indigenous Australians, who are genetically related to PNG highlanders [121].

Open questions related to the burden of TB in PNG

The extent of the current TB epidemic in PNG remains largely unknown und epidemiological data is scarce. There is some anecdotal evidence suggesting that the prevalence of pulmonary TB would be lower in the highlands than in the lowlands of PNG, but further investigations are needed to confirm this difference and identify the potential reasons for it. Also to be explored are the potential explanations for the observed large difference in drug resistance rates observed between the Western Province (25.0% MDR-TB [102]) and the Madang region (5.2% MDR-TB). One reason

could be that the proximity of Australian health facilities may have biased the study population in Western Province towards sicker individuals, potentially seeking better treatment in Australian territories, where second-line drugs are more readily available (Coulter C., personal communication). Differences in TB prevalence or in MDR-TB rates are to be expected throughout PNG and data from multiple sites would provide the necessary baseline information to investigate these aspects.

Learning from the limitations of the present study (see section above), future investigations could include active case finding and thereby increase access to TB diagnosis among populations at risk. Indeed, active case finding approaches can contribute to reduce the delay before treatment initiation, when the risk of *M. tuberculosis* transmission to susceptible contacts is the highest; a risk which can be particularly enhanced in developing countries, where a larger fraction of *M. tuberculosis* infected people tends to develop active TB [57].

The future of TB control in PNG

Efforts need to be sustained to achieve a long term decrease of the TB burden in PNG. In addition to the monitoring of treatment success performed in the frame of the treatment cohort study in Madang, stronger investigations about TB history and potential transmission foci would provide valuable information to identify where future efforts should be put to better curb TB in PNG.

Recently, Ongugo *et al.* reviewed the possible drawbacks that could impair the success of DOTS in PNG [122]. According to the authors, it is important that the PNG government better supports the extension of DOTS with a particular attention on overcrowded settlements, which are prone to TB outbreaks and on rural areas, where access to health care is limited due to the geographical constrains. An increased rate of disease detection is also particularly important and can only be achieved if good diagnostic tools are available together with well trained staff to perform the diagnosis adequately. In parallel, TB awareness is essential among the general community and among medical workers in order to help reducing delays between symptom onset and TB diagnosis, thereby decreasing the risks of transmission. Furthermore, the directly observed therapy aims at limiting adherence problems and should be particularly encouraged upon anti-TB treatment initiation. Fixed-dose drug combinations are also a way of increasing compliance by reducing the pill burden and should be considered whenever possible. But importantly, Ongugo *et al.* recommended that reliable first- and second-line drugs should be available in a controlled way among clinics throughout the country [122]. Coupled to prompt resistance testing, this should prevent the expansion of MDR-TB in PNG. Nevertheless, the future of TB control may not only

concern clinical interventions but may also need to integrate economic, social and educational actions in a comprehensive approach aiming at halting the spread of TB in PNG [123].

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Appendix

Mycobacterium algericum sp. nov., a novel rapidly growing species related to *Mycobacterium terrae* complex and associated with goat lung lesions

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Mycobacterium algericum sp. nov., a novel rapidly growing species related to the *Mycobacterium terrae* complex and associated with goat lung lesions

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A previously undescribed, rapid-growing, non-chromogenic *Mycobacterium* isolate from a goat lung lesion in Algeria is reported. Biochemical and molecular tools were used for its complete description and showed its affiliation to the *Mycobacterium terrae* complex. 16S rRNA, *rpoB* and *hsp65* gene sequences were unique. Phylogenetic analyses showed a close relationship with *M. terrae sensu stricto* and *Mycobacterium senuense*. Culture and biochemical characteristics were generally similar to those of *M. terrae* and *M. senuense*. However, in contrast to *M. terrae* and *M. senuense*, the isolate was positive for urease production and had faster growth. The mycolic acid profile was distinct from those of *M. terrae* and *M. senuense*, thus further supporting the new taxonomic position of the isolate. We propose the name *Mycobacterium algericum* sp. nov. for this novel species. The type strain is TBE 500028/10^T (=Bejaia^T=CIP 110121^T=DSM 45454^T).

Non-tuberculous mycobacteria are typically found in the environment, but can be the cause of occasional opportunistic infections in humans or animals (Tortoli, 2009).

Members of the *Mycobacterium terrae* complex (*Mycobacterium terrae sensu stricto*, *Mycobacterium non-chromogenicum*, *Mycobacterium triviale* and *Mycobacterium senuense*) are ubiquitous and potentially opportunistic pathogens. Indeed, some reports showed potential human pathogenicity (Krisher *et al.*, 1988; Mayo *et al.*, 1998; Smith *et al.*, 2000). Disseminated *M. terrae* infections have also been reported among AIDS patients (Carbonara *et al.*, 2000). *M. senuense* was recently described as a novel *Mycobacterium* species closely related to the *M. terrae* complex (Mun *et al.*, 2008). It was isolated from a Korean patient with a symptomatic pulmonary infection.

In this report, we present a previously undescribed *Mycobacterium* species, for which we propose the name *Mycobacterium algericum* sp. nov. (type strain, TBE 500028/10^T). Previous publications have shown that phenotypic and biochemical analyses often do not provide an accurate identification of *Mycobacterium* species (Cloud *et al.*, 2006; Lee *et al.*, 2004; Mun *et al.*, 2008; Springer *et al.*, 1996). Therefore, using a combination of both biochemical and molecular techniques, we showed that isolate TBE 500028/10^T, collected from a pulmonary lesion on a goat in Algeria, was representative of a novel *Mycobacterium* species related to the *M. terrae* complex, in particular *M. terrae sensu stricto* and *M. senuense*.

The isolate was collected in the Bejaia department (Wilaya) in Algeria in 2008, at the slaughterhouse of Souk El Tenine. Lung lesions observed on goat carcasses were sampled and sent to the Pasteur Institute in Alger. There, Ziehl–Neelsen

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *hsp65* and partial *rpoB* gene sequences of strain TBE 500028/10^T are GU564404, GU564405 and GU564406, respectively.

A supplementary table is available with the online version of this paper.

staining and microscopy were performed. After grinding and decontamination by Petroff's method (4 % NaOH) (Ghosh *et al.*, 1978; Petroff, 1915), samples were inoculated on Löwenstein–Jensen growth medium. Tubes were incubated at 37 °C (ambient atmosphere) and monitored weekly until colonies suggesting mycobacterial growth were observed. Tubes with potential mycobacterial colonies were sent to Switzerland for further biochemical and molecular characterization of the isolate.

Biochemical characterization of strain TBE 500028/10^T was performed by observing growth under various conditions, according to methods described by Murray *et al.* (2007); the determined characteristics were compared with those of the closely related *Mycobacterium* species *M. terrae* and *M. senuense*. To determine the optimal growth temperature, Middlebrook 7H10 agar plates were inoculated and incubated at temperatures ranging from 25 °C (with and without light) to 45 °C (ambient atmosphere). We also tested growth ability on MacConkey agar with and without 5 % NaCl at 37 °C (ambient atmosphere) and tellurite reduction, as well as nitrate reductase, urease, catalase and pyrazinamidase production. Colony morphology and pigmentation are described. Cell morphological analyses were conducted after growth in 7H9 liquid broth with 10 % OADC supplement (oleic acid, bovine albumin, glucose and catalase). Cells were harvested in the exponential-growth phase and stained with auramine–rhodamine. Microscopic imaging was performed on a Zeiss Axiovert 200M microscope using a 100-fold magnifying objective (Carl Zeiss). Cell dimensions were determined by using the measurement tool provided by the SlideBook version 4.1 software (Intelligent Imaging Innovations). Acid–alcohol fastness was assessed by Ziehl–Neelsen staining.

Susceptibility to the antibiotics rifampicin (1.0 and 10.0 mg l^{-1}), rifabutin (0.1 and 1.0 mg l^{-1}), ethambutol (5.0 and 50 mg l^{-1}), clarithromycin (4.0, 16.0, 32.0 and 64.0 mg l^{-1}), amikacin (1.0 and 10 mg l^{-1}), ofloxacin (2.0 and 10.0 mg l^{-1}) and moxifloxacin (0.5, 2.5 and 10 mg l^{-1}) was tested in mycobacterial growth indicator tubes (MGIT 960; Becton Dickinson) and monitored with EpiCenter software and the TB eXiST module (Springer *et al.*, 2009).

HPLC analyses were performed to investigate the profile of cell-wall mycolic acids (Butler & Guthertz, 2001; CDC, 1996). The profile obtained for our isolate was compared with those from other closely related *Mycobacterium* species.

DNA was extracted from cultures using the Bio-Rad InstaGene Matrix. In order to assess the phylogenetic relationships of the isolate, we performed sequencing of the nearly complete 16S rRNA gene (1521 bp, corresponding to *Mycobacterium tuberculosis* H37Rv^T *rrs* nt 18–1534) using previously described primers and protocols (Rogall *et al.*, 1990; Springer *et al.*, 1996) and two new primers (16s_742_fw, 5′-AGCGTGGGGAGCGAACAGG-3′; 16s_1408_rv, 5′-CCCGAAGCCGGTGGCCTAA-3′). Two regions of the *rpoB* gene were amplified and sequenced: 710 bp (*M. tuberculosis* H37Rv^T *rpoB* nt 2436–3145) according to Adékambi *et al.* (2003), and 553 bp (*M. tuberculosis* H37Rv^T *rpoB* nt 1092–1645) according to Kim *et al.* (1999), with a modified reverse primer: MR2, 5′-CAGCTCG-TCGTCGTCCTC-3′. A 441 bp part of the *hsp65* gene (*M. tuberculosis* H37Rv^T *groEL2* nt 145–585) was sequenced according to Telenti *et al.* (1993). All amplifications included the reference strain *M. tuberculosis* H37Rv^T as positive control, and buffers without any added mycobacterial DNA as negative control.

Nucleotide sequences were obtained for both forward and reverse primers. Sequence contingency alignment was performed for each gene with the software SeqMan version 7.0 (DNASTAR Inc.). The obtained consensus sequences were compared with mycobacterial sequences by similarity search in GenBank, using the BLASTN algorithm. Phylogenetic trees comparing our isolate with other *Mycobacterium* species were obtained for each gene by using the MEGALIGN software (DNASTAR Inc.) with default settings for CLUSTAL V neighbour-joining multiple alignments. Note that the *rpoB* phylogeny was calculated based on the 553 bp sequence fragment. All obtained trees were established by bootstrap analyses with 1000 resamplings and 111 seeds.

The goat from which the isolation was made presented nodular lung lesions of caseous aspect. Direct microscopy of Ziehl–Neelsen-stained tissues showed low-density (six acid–alcohol-resistant bacilli in 300 observed fields) infections with slightly curved bacilli. The isolate was inoculated into Löwenstein–Jensen tubes in Algeria. Growth rate was slow on Löwenstein–Jensen medium: small, round, white–yellow colonies were observed after 35 days. Inversely, subculture on Middlebrook 7H10 agar plates was rapid; microcolonies could be observed after 1 week at temperatures ranging between 25 and 40 °C, with or without light. Optimal growth was seen at 37 and 40 °C, whereas no growth was observed at 45 °C. On Middlebrook 7H10 agar plates, colonies were small, polymorphic, white, non-chromogenic, smooth and with irregular edges. Ziehl–Neelsen and auramine–rhodamine staining showed acid-fast, 1.4 ± 0.2 μm long, rod-shaped bacilli. No spores, cords or filaments were observed.

No growth was observed on MacConkey agar with or without 5 % NaCl. Production of urease, a positive nitrate reductase reaction, a positive catalase reaction at 25 °C, and tellurite reductase activity were observed.

Drug-susceptibility testing in MGIT 960 showed susceptibility to all drugs tested (see Supplementary Table S1, available in IJSEM Online), with the exception of low-level resistance to aminoglycosides (1.0 mg amikacin l^{-1}), quinolones (0.5 mg moxifloxacin l^{-1} and 2.0 mg ofloxacin l^{-1}) and rifampicin (1.0 mg l^{-1}). Pyrazinamidase production was detected. Strain TBE 500028/10^T is thus sensitive to pyrazinamide.

HPLC analysis of the cell wall's mycolic acids demonstrated a unique profile. Although related closely to the profiles

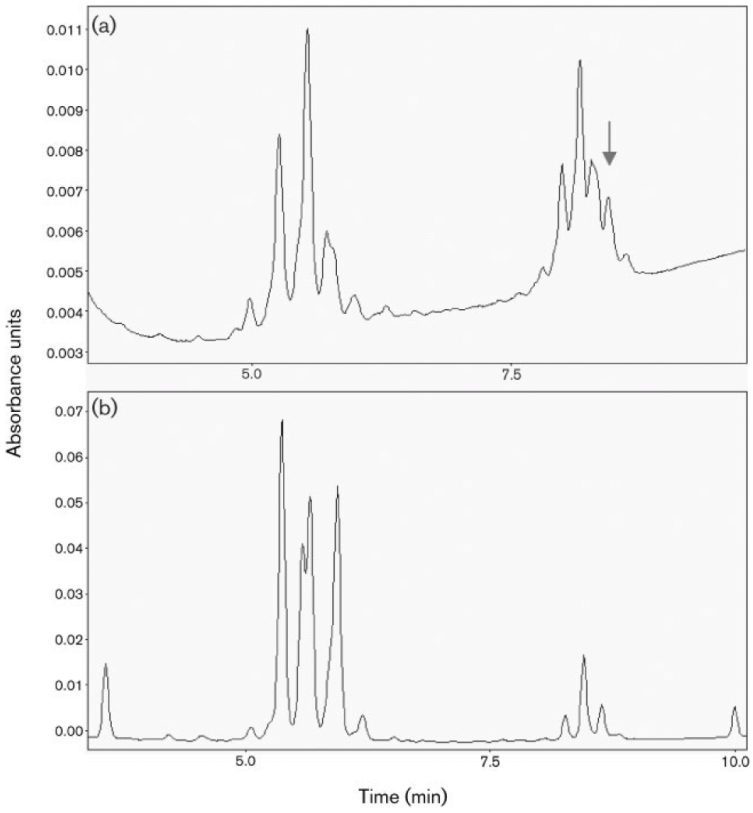

Fig. 1. Mycolic acid profiles obtained by HPLC. (a) TBE 500028/10^T; (b) *M. terrae* ATCC 15755^T. The arrow in (a) indicates an additional peak in the late cluster of the profile of strain TBE 500028/10^T.

observed for other *M. terrae* complex subspecies, the chromatogram obtained for our isolate distinctively presented an additional peak in the late cluster (Fig. 1).

The overall biochemical profile of the isolate was similar to those of *M. terrae* and *M. senuense*, with the exception of an intermediate instead of a slow growth rate at 37 °C (Table 1). Furthermore, in contrast to the type strains of *M. terrae* and *M. senuense*, strain TBE 500028/10^T had a positive urease activity and a different antibiotic-susceptibility pattern (Mun *et al.*, 2008; Murray *et al.*, 2007; Smith *et al.*, 2000). Indeed, unlike strain TBE 500028/10^T, *M. terrae* and *M. senuense* are susceptible to rifampicin, amikacin and quinolones (*M. senuense* only).

Based on the BLAST sequence match obtained for the 16S rRNA gene (GenBank accession no. GU564404), strain TBE 500028/10^T was shown to be most closely related to *M. senuense* 05-832^T (1492/1522 nt, 98.0 %), *M. terrae* ATCC 15755^T (1451/1470 nt, 98.7 %) and *Mycobacterium arupense* AR30097^T (1458/1495 nt, 97.5 %). The two combined partial sequences of the *rpoB* gene (GenBank accession no. GU564406) showed that strain TBE 500028/10^T was related most closely to *M. senuense* 05-832^T (403/421 nt, 95.7 %), *M. terrae* ATCC 15755^T (591/633 nt, 93.4 %), *Mycobacterium confluentis* CIP 105510^T (632/703 nt, 89.9 %) and *Mycobacterium chitae* CIP 105383^T (632/703 nt, 89.9 %). The *hsp65* gene sequence (GenBank accession no. GU564405) showed that our strain was related most closely to *M. senuense* strain DSM 44999^T (421/431 nt, 97.7 %) and *M. terrae* strain CIP 104321^T (414/426 nt, 97.2 %).

Table 1. Cultural and biochemical characteristics for *M. algericum* sp. nov. in comparison with the closely related *M. terrae* and *M. senuense*

Taxa: 1, *M. algericum* TBE 500028/10^T; 2, *M. terrae* ATCC 15755^T; 3, *M. senuense* DSM 44999^T. Data for *M. terrae* and *M. senuense* were retrieved from the literature (Mun *et al.*, 2008; Murray *et al.*, 2007). +, Positive; −, negative; +/−, variable; I, intermediate in roughness; S, smooth; W, white; Y, yellow. All taxa were non-photochromogenic, negative for growth at 45 °C and on MacConkey agar both with and without 5 % NaCl, and positive for production of nitrate reductase, catalase, tellurite reductase and pyrazinamidase.

Characteristic	1	2	3
Growth within 7 days at 37 °C	+	+/−	−
Optimal growth temperature (°C)	37–40	35	37
Colony morphology on 7H10	IW	IWY	SWY
Urease production	+	−	−

The close relationship to *M. terrae* and *M. senuense* was confirmed by phylogenetic analyses (Fig. 2). Trees based on *rpoB* and *hsp65* gene sequences each showed similar groupings for strain TBE 500028/10^{T}, but slightly different from that of the 16S rRNA gene tree. In the *rpoB* and *hsp65* trees, TBE 500028/10^{T} forms a branch dependent of *M. terrae* together with *M. senuense*, whereas in the 16S rRNA gene tree, strain 500028/10^{T} forms a branch directly

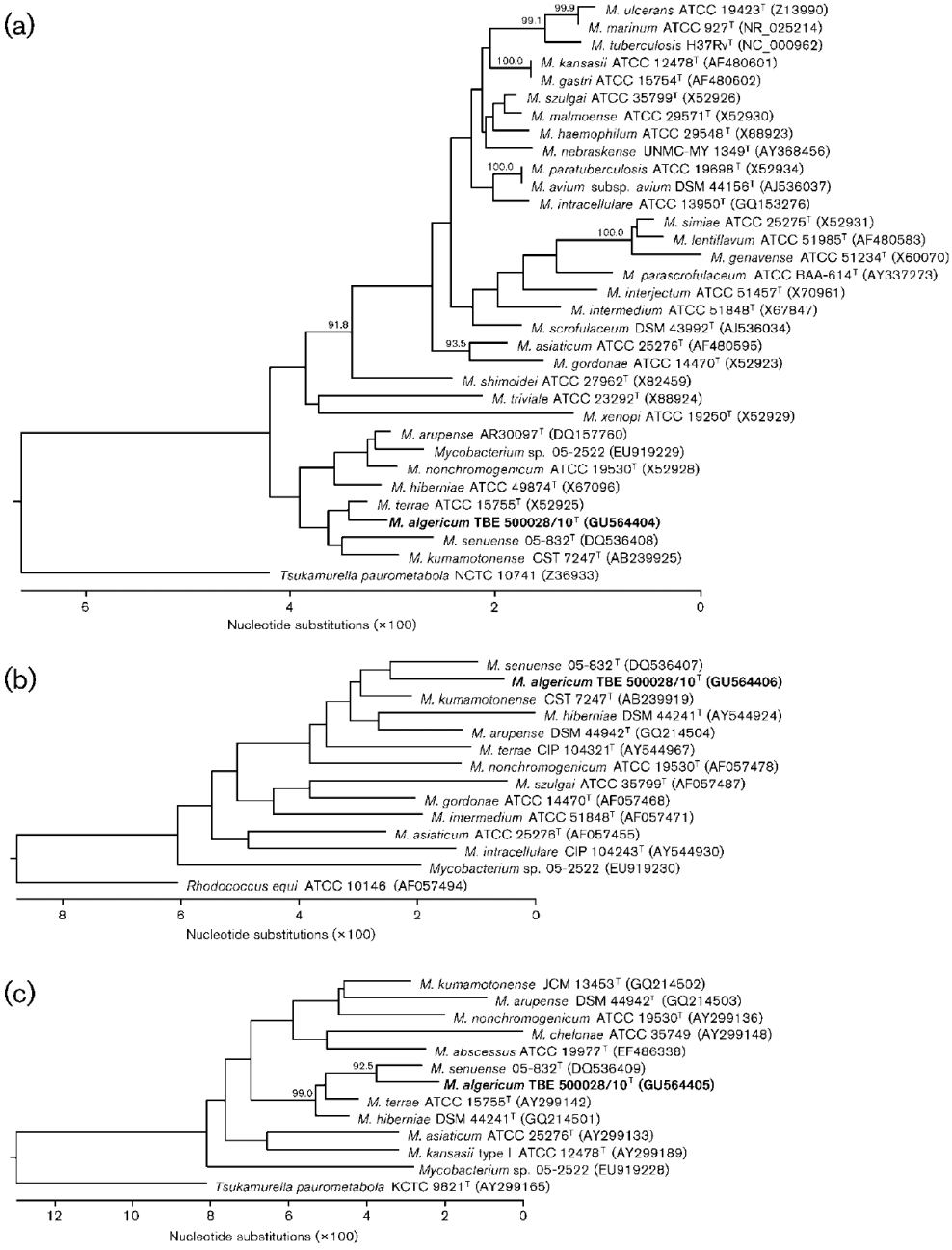

Fig. 2. Phylogenetic trees designed with the neighbour-joining method and bootstrapped 1000 times. Bootstrapping values >90% are indicated at nodes. Trees were based on: (a) 16S rRNA gene; (b) 553 bp partial sequence of the *rpoB* gene; (c) *hsp65* gene.

dependent from *M. terrae*, with *M. senuense* being situated in another cluster. These topologies were also supported by high bootstrap values.

The species which our isolate resembles most closely, *M. terrae* and *M. senuense*, are both slow-growing, non-chromogenic and potentially pathogenic to humans.

Description of *Mycobacterium algericum* sp. nov.

Mycobacterium algericum (al.ge′ri.cum. N.L. neut. adj. *algericum* pertaining to Algeria, the country where the strain was first isolated).

Bacillus that stains acid–alcohol-fast. Cells are rod-shaped and 1.4±0.2 μm long. Cording, spores and filaments are not observed. On Middlebrook 7H10 agar plates, growth is fast, with optimal growth between 37 and 40 °C. Microcolonies can be observed after 1 week. However, 5 weeks are necessary to observe colonies on Löwenstein–Jensen medium. Colonies on Middlebrook 7H10 agar plates are small, polymorphic, white, non-chromogenic, smooth and with irregular edges. Colonies grown on Löwenstein–Jensen medium are small, round and white–yellow. No growth is observed on MacConkey agar plates with or wthout 5 % NaCl. Urease, nitrate reductase, catalase and tellurite reductase production is positive. Pyrazinamidase production is positive. Susceptible to clarithromycin, ethambutol and rifabutin, but low-level resistant to amikacin, moxifloxacin, ofloxacin and rifampicin. HPLC analysis of mycolic acids showed a unique profile. Genetically, 16S rRNA, *rpoB* and *hsp65* gene sequences are unique. Phylogenetic analyses showed that the species is related to *M. terrae sensu stricto* and *M. senuense*.

The type strain is TBE 500028/10^T (=Bejaia^T=CIP 110121^T=DSM 45454^T), isolated from a lung lesion of an Algerian goat.

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